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Fish Physiology, Fish Toxicology, and Fisheries Management:

Proceedings of an International Symposium, Guangzhou, PRC, September 14-16, 1988

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FISH PHYSIOLOGY, FISH TOXICOLOGY, AND FISHERIES MANAGEMENT

Proceedings of an International Symposium,
Guangzhou, PRC, September 14-16, 1988

Edited by

Robert C. Ryans

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FOREWORD

Joint ecological research involving scientists from every country in the world is essential if global environmental problems are to be solved with international expertise. Recognition of this international aspect of environmental pollution is reflected in the joint activities undertaken under Annex 3, Item 4 of the USA-PRC Protocol for Environmental Protection. This component of the protocol provides for cooperative research on the environmental processes and effects of pollution on freshwater organisms, soils, and groundwater and on the application of transport and transformation models.

Specific areas of cooperation in environmental research include inorganic chemical characterization and measurement; inorganic chemical transport and transformation process characterization; biological degradation process characterization; oxidation/reduction process characterization; field evaluation of selected transport, exposure and risk models; and application of models for environmental decision-making concerning organic pollution in semi-arid conditions, heavy metal pollution, and permissible loading of conventional and toxic pollutants in Chinese rivers. Activities include seminars, workshops, joint symposia, training programs, joint research, and publications exchange.

The first symposium presented under this portion of the protocol was held on the campus of Zhongshan University, Guangzhou, PRC, on September 14-16, 1988. Sponsored jointly by the National Science Foundation of the PRC, Zhongshan University, the U.S. Environmental Protection Agency, the Academy of Sciences of China, the Canadian Society of Zoology, and the American Fisheries Society, the symposium attracted presentations from eight countries who recognized the need for knowledge exchange on a global scale.

Symposia are an effective means of fostering cooperation among scientists from different countries as environmental organizations seek to efficiently gain the information necessary to predict the effects of pollutants on ecosystems and apply the results on a global scale. The symposia provide a forum through which distinguished scientists from laboratories and institutes from several countries can exchange scientific expertise on environmental problems of concern to EPA and the international environmental community.

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ABSTRACT

Scientists from eight countries presented papers at the first International Symposium on Fish Physiology, Fish Toxicology, and Fisheries Management, which was held on the campus of Zhongshan University, Guangzhou, PRC. This proceedings includes 30 papers presented in sessions on the reproduction and growth of fish; on the physiology, behavior and genetics of fish; and on toxicology and risk assessment in aquatic systems. Papers address hormone functions, sex manipulation in aquaculture, cloning and induced breeding in fish, calcium and phosphorus requirements, fish diets and metabolism, and utilization of inorganic nitrogen. Descriptions are provided concerning research on gas transfer, gill ventilation control, carbon dioxide effects, ammonia effects, environmental acidification effects, phototaxis, sex pheromones effects, gonad maturation, antifreeze protein genes, and bioenergetics modeling. Presentations also covered fish conservation, water quality protection, predictions of chronic effects levels, chemical toxicity, and toxicant modeling.

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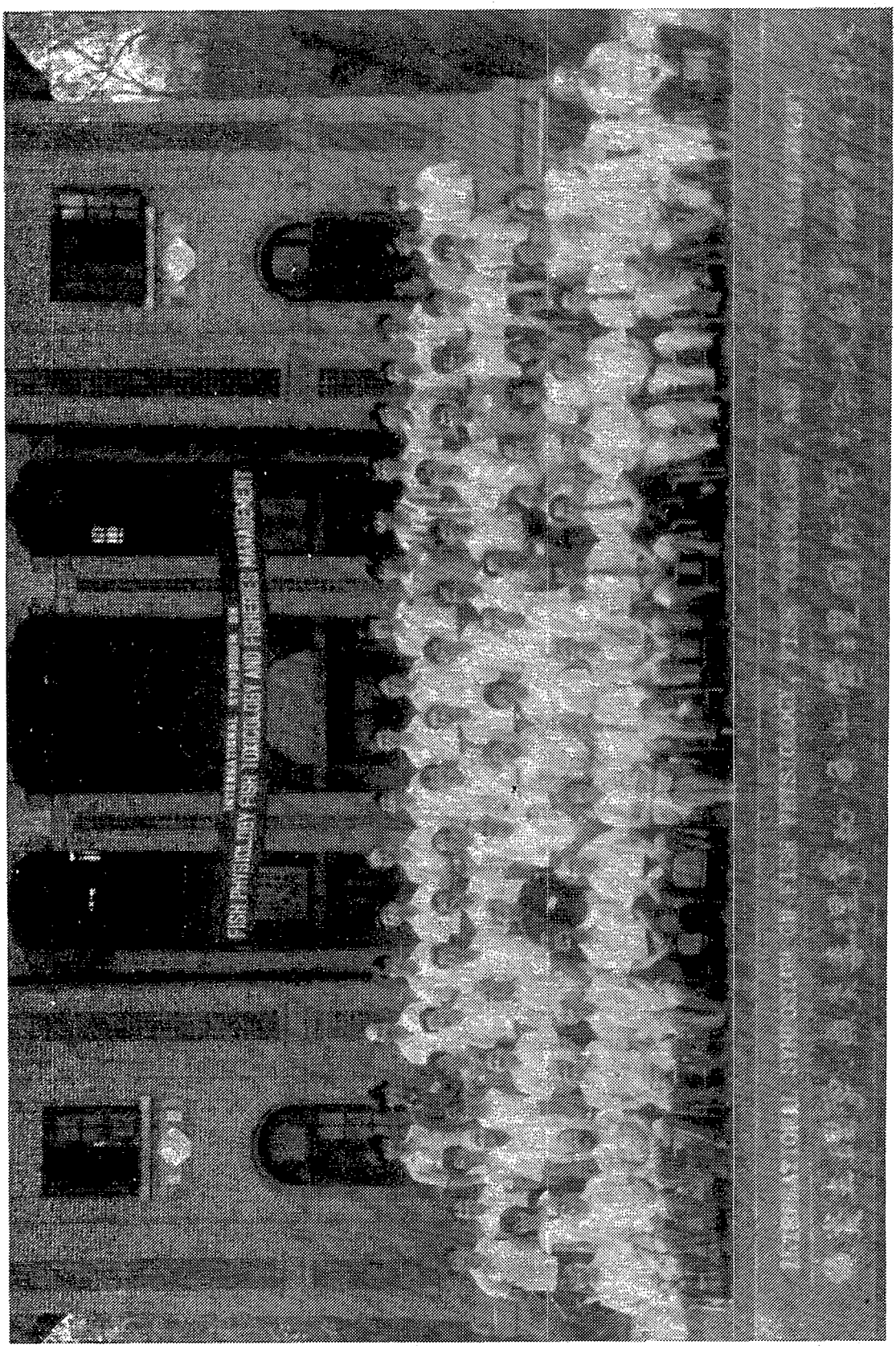

STRUCTURE AND FUNCTION OF GONADOTROPIN-RELEASING HORMONES IN FISHES

by

Nancy M. Sherwood[1]

ENVIRONMENT, BRAIN, AND GnRH

The brain is an important organ for the control of reproduction in all vertebrates including the most primitive fishes. In the brain, a number of factors are produced that may affect reproduction, but the factor with the most direct control is gonadotropin-releasing hormone (GnRH). In fishes as in other vertebrates, this hormone is a peptide of ten amino acids synthesized in a few specialized nerve cells. One function of GnRH after it is released from the neurons is stimulation of the pituitary to synthesize and release gonadotropin hormones. These pituitary hormones, in turn, activate the gonads. Hence, GnRH is essential for internal control of reproduction. This decapeptide also may be a critical node for some environmental effects on reproduction in fishes. A number of environmental factors that act on the nervous system through the senses may inhibit the release of GnRH. One supporting argument for the relationship between the environment and GnRH inhibition is that administration of exogenous GnRHs has been successful for the induction of breeding in cultured fish that will not spawn in captivity.

SEVERAL FORMS OF GnRH IN FISHES

GnRH has been detected in a variety of vertebrates from lamprey to humans. The evidence to date suggests the hormone is always ten amino acids with the same end groups, but the individual amino acids within the molecule vary. The primary structure of GnRH has been determined for the five mole cules shown below (Burgus et al. 1972; King and Millar 1982a,b; Matsuo et al. 1971; Miyamoto et al. 1982, 1983, 1984; Sherwood et al. 1983, 1986a). Part of the structure of the molecule has been retained for millions of years. The conserved portion is underlined and includes the amino acids 1, 2, 4, 9, 10, and the C-terminal amide.

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	1	2	3	4	5	6	7	8	9	10
Lamprey	pGlu-	His-	Tyr-	Ser-	Leu-	Glu-	Trp-	Lys-	Pro-	Gly-NH2
Salmon	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Trp-	Leu-	Pro-	Gly-NH2
Chicken II	pGlu-	His-	Trp-	Ser-	His-	Gly-	Trp-	Tyr-	Pro-	Gly-NH2
Chicken I	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Gln-	Pro-	Gly-NH2
Mammal	pGlu-	His-	Trp-	Ser-	Tyr-	Gly-	Leu-	Arg-	Pro-	Gly-NH2

Other forms of GnRH also exist, but their primary sequence is not yet known. These other forms can be detected, however, with cross-reacting antisera that were made against one of the known forms. Many of these immunoreactive (ir) GnRHs can be separated from one another by chromatography, such as high performance liquid chromatography (HPLC). The conclusion of these studies is that fishes contain several forms of GnRH, some of which are shared with higher vertebrates (Table 1). Also, more than one form of GnRH can exist in a particular fish species and, indeed, this is the common pattern with only a few exceptions.

DISTRIBUTION OF GnRHs IN FISHES

In addition to the salmon and lamprey forms of GnRH shown above, there are several other forms, outlined in Table 1, for the fish studied to date. The evidence varies, however, in strength and this is indicated. For some peptides, the primary structure has been determined (++++), but for others only the HPLC elution position and cross-reactivity with a nonspecific GnRH antiserum have been observed (+).

A partial phylogenetic map for GnRH has begun to emerge from the distribution studies to date. Lamprey GnRH has not been detected in other fishes. A chicken II-like form of GnRH is present in cartilaginous fish and in small amounts in many bony fishes, but the phrase "chicken II-like" means only that the molecules react with a nonspecific GnRH antiserum and have the same HPLC mobility as chicken II GnRH. It is not known whether the structures are identical with chicken II GnRH or among the fishes. The mammalian form of GnRH has been detected in primitive bony fish with a GnRH antiserum specific for the mammalian form. It also has been observed with a nonspecific GnRH antiserum (cross reacts with more than one form of GnRH) in two teleosts (eel and winter flounder), but otherwise does not appear to be present in teleosts studied to date. Rather, the mammalian form is detected in tetrapods such as amphibians and man. Finally, the salmon form is present in the teleosts studied to date except for the African catfish, which appears to have its own form. The chicken I-like form has been reported for only three species of teleosts. Other forms also may be present in fish species not yet studied.

TABLE 1. GnRH IN FISHES

Species	GnRH form							Ref.
	L-I	L-II	S	C-II	C-I	M	Novel	
hagfish (*Eptatretus stouti*)	-	-	-	-	-	-	-	1
lamprey (*Petromyzon marinus*)	++++	+++						2
ratfish (*Hydrolagus colliei*)				+				3
dogfish (*Squalus acanthias*)				+				1
dogfish (*Poroderma africanum*)			+	+				4
reedfish (*Calamoichthys calabaricus*)						++		5
sturgeon (*Acipenser transmontanus*)				+		++		5
alligator gar (*Lepisosteus spatula*)				+		++		5
bowfin (*Amia calva*)						+		6
moray eel (*Gymnothorax fimbriatus*			+			+	+	7
herring (*Clupea harengus pallasi*)			+	+				8
milkfish (*Chanos chanos*)			+	+				9
goldfish (*Carassius auratus*)			+	+				10,11
catfish (*Clarias gariepinus*)				+			+	12
salmon (*Oncorhynchus keta*)			++++	+				13,14
trout (*Salmo gairdneri*)			+	+				15
hake (*Merluccius capensis*)			+		+			4
codfish (*Gadus morhua morhua*)			+++					16
molly (*Poecilia latipinna*)			+					17
snook (*Centropomus undecimalis*)			+				+	17
sea bass (*Centropristis striatus*)			+					17
mullet (*Mugil cephalus*)			+	+				9
tilapia (*Tilapia sparrmanii*)			+		++			4
wrasse (*Coris julis*)			+					4
winter flounder (*Pseudo-pleuronectes americanus*)			+		+	+		18

(1) Sherwood & Sower, 1985; (2) Sherwood et al., 1986a; (3) Lovejoy & Sherwood, 1989; (4) Millar & King, 1987; (5) Sherwood et al. 1988a; (6) Crim et al., 1985; (7) Shih et al., 1988; (8) Sherwood, 1986; (9) Sherwood et al., 1984; (10) Sherwood & Harvey, 1986; (11) Yu et al., 1988; (12) Sherwood et al., 1989; (13) Sherwood et al., 1983; (14) Sherwood, 1987b; (15) Sherwood et al., 1986b; (16) Wu et al., 1986; (17) Sherwood & Grier, unpubl.; (18) Idler & Everard, 1987. Table 1 modified from Sherwood & Lovejoy, *Fish Physiol. Biochem.* (in press). Reprinted with permission.

STRUCTURAL VARIATION OF GnRH WITHIN A SINGLE SPECIES OF FISH

Table 1 shows that most fish species have more than one form of GnRH. The amino acid composition of the minor form (II) of GnRH in lamprey is known and differs in three amino acids from the major form (I). The other second forms of GnRH in fishes have been identified by immunological and chromatographic methods, but it is known that the second form is not a metabolic product that has lost its amide or been cleaved because such degraded forms are not recognized by some of the antisera. The structures of the second forms need to be determined by sequencing of the amino acids or DNA nucleotide bases. The quantity of GnRH in the brain is very small so that analysis is done on pools of brains. Therefore, it also is not known whether multiple forms of GnRH are in individual fish or in the pools of fish. The relative quantities of the various forms of GnRH in a species can be measured accurately only by amino acid analysis; immunological measurements vary greatly depending on the percent cross-reactivity of an antiserum with a particular form of GnRH. In goldfish, for example, the quantity of the two GnRH forms varied considerably in the radioimmunoassays (RIA) depending on the form of GnRH that was used for antiserum production, RIA tracer, and RIA standard (Yu et al. 1988).

RELATIONSHIP OF GnRH STRUCTURE AND RELEASING FUNCTION

Alterations in the GnRH molecule change its potency for releasing gonadotropins and its binding affinity to the pituitary receptor. Amino acids 1-3, especially His^2 and Trp^3, are essential for the release of gonadotropins (GtH), whereas amino acids 4-10 are important for receptor binding in mammals (Burgus et al. 1973, Schally and Coy 1977, Rivier et al. 1981). Analogs with substitutions in 2 and 3 positions bind competitively with the pituitary receptor, but lack releasing ability.

The native forms of the five GnRHs have been compared for potency in only a few species of fish. Lamprey GnRH was shown indirectly to release gonadotropins in lamprey; changes in the gonadal steroid levels were measured (Sower et al. 1987) because the lamprey gonadotropins have not been characterized for use in RIA.

The effect of lamprey GnRH has been observed in the goldfish where the peptide was equipotent with the salmon and mammalian forms *in vivo* (Peter et al. 1985, 1987). The other forms of GnRH show considerable overlap in potency in the various fish tested. Zohar et al. (1989) found that mammalian and salmon GnRH were equally effective both *in vivo* and *in vitro* in the seabream, *Sparus aurata*. Likewise, Van Der Kraak et al. (1987) found the same two peptides were equally effective *in vivo* in salmon. Salmon GnRH was 10 times more potent than mGnRH, however, as a threshold dose for release of gonadotropin from pituitary fragments in goldfish (MacKenzie et al. 1984).

In the sterlet fish, which normally do not ovulate in captivity, mammalian and chicken I GnRH did not advance ovulation, whereas salmon GnRH induced 30% to ovulate (Horvath et al. 1986). Salmon and mammalian GnRH (100 ug/kg), but not chicken I or II GnRH, resulted in elevated gonadotropin levels

in testosterone-treated juvenile trout (L.W. Crim, pers. comm.). Further testing of the native forms of GnRH including the lamprey form are needed in fish species that are quite separate in evolutionary terms. But the mammalian, salmon and chicken forms appear to show more overlap of potency in fish compared with mammals.

Part of the explanation for differing potencies of molecular forms of GnRH in a particular species may be their affinities for the species receptor. The native GnRHs differ primarily in positions 5-10 so that variation in receptor binding is predicted. Observations support the prediction, in that binding affinity of GnRHs with rat anterior pituitary membranes is 100% for mammalian GnRH, but only 13% for chicken II GnRH, 5% for salmon GnRH, and less than 1% for chicken I and lamprey (Millar et al. 1986, Sherwood et al. 1983). Receptor binding studies in the fish pituitary have been reported recently for goldfish (Habibi et al. 1987), catfish (De Leeuw et al. 1988), and winter flounder (Crim et al. 1988). Comparison of mammalian GnRH and salmon GnRH in winter flounder showed they were equally effective in displacing a GnRH analog bound to the pituitary receptors. Most deductions about nonmammalian GnRH receptors, however, are based on comparison of releasing potency among native GnRHs. Information on the relative potency and binding affinity for each GnRH is needed for different fish species to determine whether structural changes have evolved in the GnRH receptors in fish.

GnRH ANALOGS AND FUNCTION

The relationships between the structure of native forms of GnRH and releasing function can be altered considerably by making artificial forms (analogs) of GnRH. Few analogs made with natural amino acids are more potent than the native forms, although [Phe7] chicken I GnRH is equal to salmon GnRH for induced ovulation in sterlets (Horvath et al. 1986) and there are five agonists made with natural L-amino acids that have activity equivalent or greater than mGnRH in releasing LH and/or FSH in rat assays (Folkers et al. 1986). A number of GnRH analogs made with unnatural or D forms of amino acids are potent in fish for release of GtH or induction of ovulation/spermiation. Two of the most active and commonly used analogs for fish are shown below.

	1 2 3 4 5 6 7 8 9 10
Mammalian GnRH	pGlu-His-Trp-Ser-Tyr-Gly -Leu-Arg-Pro-Gly-NH2
agonistic analog	pGlu-His-Trp-Ser-Tyr-<u>D-Ala</u>-Leu-Arg-Pro-<u>NH-CH2-CH3</u>
Salmon GnRH	pGlu-His-Trp-Ser-Tyr-Gly -Trp-Leu-Pro-Gly-NH2
agonistic analog	pGlu-His-Trp-Ser-Tyr-<u>D-Arg</u>-Trp-Leu-Pro-<u>NH-CH2-CH3</u>

Other analogs that are more active than the native GnRHs in fish (see Crim et al. 1987, Crim. (pers. comm.), Horvath et al. 1986, Van Der Kraak et al. 1987, Zohar et al. 1989) are shown in Table 2.

TABLE 2. ACTIVE GnRH ANALOGS IN FISHES.

Type of GnRH analog		
Mammalian analogs	pGlu-His-Trp-Ser-Tyr-D-Trp	-Leu-Arg-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Leu-Arg-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Leu	-Leu-Arg-Pro-Aza-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Nal(2)	-Leu-Arg-Pro-Aza-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Trp	-Leu-Arg-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-Ala	-Leu-Arg-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-TLe	-Leu-Arg-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Leu-Arg-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-Ser(Bu^t)	-Leu-Arg-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-His-(imBzl)	-Leu-Arg-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-Nal(2)	-Leu-Arg-Pro-NH-CH2-CH3
Salmon analogs	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Trp-Leu-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Phe	-Trp-Leu-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Ala	-Trp-Leu-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-Arg	-Trp-Leu-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-Trp	-Trp-Leu-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Trp-Leu-Pro-NH-CH2-CH3
Chicken I analogs	pGlu-His-Trp-Ser-Tyr-D-Trp	-Leu-Gln-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-hArg(Et_2)	-Leu-Gln-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Phe	-Leu-Gln-Pro-Gly-NH2
	pGlu-His-Trp-Ser-Tyr-D-Phe	-Leu-Gln-Pro-NH-CH2-CH3
	pGlu-His-Trp-Ser-Tyr-Gly-	-Phe-Gln-Pro-NH-CH2-CH3
Chicken II analogs	pGlu-His-Trp-Ser-His-D-Arg	-Trp-Tyr-Pro-Gly-NH2
	pGlu-His-Trp-Ser-His-D-hArg(Et_2)	-Trp-Tyr-Pro-Gly-NH2

The analogs appear to be more resistant to enzymatic breakdown (Zohar et al. 1989) and hence are effective in the body for a long time. They either bind with higher affinity or are more resistant to degradation. In practical terms, the analogues are long lasting and a number of them, regardless of

whether fashioned on the backbone of the salmon, mammalian or chicken GnRHs, are effective in fish. Antagonistic GnRH analogs have been made for mammals, but few for fish. An example of an antagonist for lamprey is shown below:

	1	2	3	4	5	6	7	8	9	10
Lamprey GnRH	pGlu-	His	-Tyr	-Ser-	Leu-	Glu	-Trp-	Lys-	Pro-	Gly-NH2
antagonistic analog	pGlu-	D-Phe	-D-Pro	-Ser-	Leu-	D-Phe	-Trp-	Lys-	Pro-	Gly-NH2

Prevention of reproduction can be a considerable advantage in fish such as lamprey that destroy commercially available fish or in teleost fish that would continue to grow if reproduction were blocked. In both cases, however, large numbers of fish would have to be treated. Administration of the peptide in water would be required, but a cost-efficient method has not been developed.

ASSESSING REPRODUCTIVE FUNCTIONS OF GnRH

The use of GnRH for induced breeding has now been tested in a variety of fish. These fish span all the categories of cold freshwater fish, cold water marine fish, warm freshwater fish, and warm water marine fish. Use of GnRH in fish from these categories is considered in detail in other papers in this symposium. To illustrate the use of GnRH analog forms, the warm water marine sea bass will be considered. The sea bass study shows the use of two different GnRH analogs and the importance of time, dose, and method of administering GnRH. Native GnRH is quickly metabolized; the half life in goldfish is only 20 minutes (Sherwood and Harvey 1986). Therefore, the method of administering the peptide is important.

The sea bass, Lates calcarifer, undergoes sex reversal from male to female at about 4 or 5 years of age. Spawning occurs in coastal waters and males probably remain there, but females return to inland fresh water after spawning. Two GnRH analogs were compared in individual sea bass pairs to determine their effectiveness in fish capable of multiple spawning (Almendras et al. 1988). The fish were reproductively mature. The two analogs were [D-Ala^6, Pro^9 ethylamide] mammalian GnRH and [D-Arg^6, Pro^9 ethylamide] salmon GnRH as shown in the section above (GnRH analogs and function). Prior to the comparison, one of the analogs was used to test the best method of administration. An injection of 60 to 100 ug of the D-Ala6 analog induced a single spawning in fish, but multiple injections spaced 24 hours apart produced 1 to 4 spawnings in individual females. The same analog also was placed in an Alzet osmotic pump and implanted intraperitoneally in selected fish. The pump was prepared so that only 9 ug of analog was released each day for 14 days. The result was that three fish spawned four times each on successive days and one fish spawned five times.

A similar effect was produced by embedding the analogs (100 ug) in a pellet made of cholesterol and cellulose. Two pellets with analog in a matrix of at least 95% cholesterol produced 1 to 4 spawnings on successive days in individual sea bass. This slow release method of GnRH administration in pellets appears to be an appropriate way to compare the structure-function relationship of analogs because a number of effects can be observed: number

of multiple spawnings/fish, fecundity, fertility rate, hatching rate and rematuration of the broodstock. The analogs were roughly equivalent in that both induced multiple spawnings in individual females. Each fish released 3 to 7 million eggs with 40 to 70% fertility and 8 to 63% hatching rate. In addition, the same sea bass that were induced to spawn on several successive days in June could be induced to have multiple spawnings again in September with 1 to 3 million eggs, 36 to 86% fertile eggs and 32 to 81% hatched larvae (Almendras et al. 1988).

In conclusion, the relationship between structure and function of GnRHs and their analogs depends on the species of fish tested, reproductive state, reproductive pattern of the fish, and the method by which the hormones are administered. Also a number of factors need to be evaluated after induction. It appears from this and other studies mentioned above that some analogs based on the structure of the mammalian, chicken, or salmon GnRHs are interchangeable in fish in terms of potency.

GnRH AND NON-RELEASING FUNCTIONS

The functions of GnRHs have been evaluated primarily by their releasing function for the gonadotropins. Recently, it has become clear that GnRH also has other functions. GnRH nerve fibers in fish are widely distributed in the brain in addition to terminating in the pituitary. GnRH receptors have been detected in nonhypothalamic regions of the brain in rats, but have not been reported for fish. The distribution of GnRH axons and receptors support the idea that GnRH may be a neurotransmitter in the brain. A separate collection of GnRH neurons near the olfactory bulb in most fish have terminal axons in the forebrain and in some species in the retina. Three suggestions for their function are (1) transduction of olfactory stimuli to reproduction behavior, (2) induction of pituitary gonadotrope maturation, and (3) modulation of vision (for reviews see Sherwood 1987a,c)

There is strong evidence that a nonmammalian form of GnRH is a neurotransmitter or modulator in the lumbar sympathetic ganglia of the bullfrog; this has not been reported for fish. Similarly, the enhancement of sexual receptivity has been reported for other vertebrates, but not yet studied in fish. The receptivity evidence was obtained by application of GnRH directly to the brain to avoid effects secondary to gonadotropin and steroid release. Finally, GnRH neurons in the midbrain have axons that travel in the spinal cord to terminate on the caudal neurosecretory neurons (Miller and Kriebel 1986). It is hypothesized that the GnRH fibers modulate the release of a secretion controlling contractility of oviducts and sperm ducts.

The story of GnRHs and their functions will not be complete until these novel functions have been elucidated for fish. The relative potency and binding to nonpituitary receptors of the GnRHs may reveal that the functions of GnRH are as diverse as the structures of this peptide family.

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MANIPULATION OF SEX IN AQUACULTURE

by

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INTRODUCTION

The vigourous pursuit of techniques for the control of sex in cultured teleosts over the last two decades saw its genesis in the publication of a significant review by Yamamoto (1969). This review laid the foundation on which our current, as yet incomplete, understanding of sex differentiation in fish is based. At the time of Yamamoto's review, there had been no report of the application of hormonal treatment or chromosome set manipulation for sex control in fish culture. In fact, Bardach et al. (1972), in their text on aquaculture, reported only on the application of interspecific hybridization for the production of monosex stocks. Since that time, much research activity has occurred both in the development of methods for the hormonal regulation of sex (for reviews, see Donaldson and Hunter 1982, Guerrero 1982, Hunter and Donaldson 1983, Pandian and Varadaraj 1987, Piferrer and Donaldson 1988) and for the genetic regulation of sex (for reviews, see Purdom 1983, 1986; Thorgaard 1983, 1986; Benfey and Donaldson 1988). Additional reviews discuss both hormonal and genetic sex control and the integration of these techniques (Stanley 1981, Yamazaki 1983, Donaldson 1986, Donaldson and Benfey 1987).

This review discusses the current status of sex control techniques focussed at specific end points, i.e., the production of all female, all male and sterile groups of fish. We will close with a summary of the current status of the application of these techniques in fish culture. The main emphasis will be on Pacific salmonids, with reference to other species where appropriate.

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PRODUCTION OF MONOSEX FEMALE STOCKS

DIRECT FEMINIZATION

This procedure involves treatment of fish during early development with a natural or synthetic estrogen (Figure 1). Yamamoto (1969) postulated that treatment must be initiated prior to visible morphological sex differentiationand continue until sex differentiation is evident. Treatment timing thus will vary between species that differentiate early and those that differentiate later. In a recent study in our laboratory in which coho salmon (Oncorhynchus kisutch) eyed eggs, alevins and fry received single immersion treatments of 400 ug/L 17β -estradiol at 10°C, the most sensitive period to estradiol, measured by the percentage of females in the resultant fry, occurred around the time of hatching (Piferrer and Donaldson 1989). By providing two or more immersion treatments at intervals of approximately 1 week at 10°C, it has been possible in experimental studies to obtain a high percentage of females in the resultant offspring (Hunter et al. 1986); however, attempts to utilize the technique at a production scale at commercial hatcheries have provided variable results. It should be noted that estrogens inhibit growth at moderate dosage levels and are toxic at higher levels.

MONOSEX FEMALE SPERMATOZOA

In many teleosts, including for example the salmonids and the carps, the female is homogametic and the male heterogametic. Thus, if monosex female, X chromosome bearing, spermatozoa are used to fertilize normal ova, all the offspring are normal females. Currently, two methods are used to produce female spermatozoa, both of which involve sex reversal of genotypic female embryos into phenotypic males and subsequent grow-out to mature phenotypic males that produce monosex female spermatozoa.

The two procedures are:

1. Masculinization of mixed sex larvae. This procedure, which was the first to be utilized on a practical scale, initially involves the masculinization (see below) of larvae of mixed sex. These masculinized larvae are then grown to sexual maturity and the spermatozoa from each phenotypic male are used to fertilize separate batches of ova. The eggs fertilized by each male are then incubated

EYED EGG/ALEVIN

ESTROGEN ↓ IMMERSION

PHENOTYPIC FEMALE

Figure 1. Direct feminization by estrogen treatment.

separately and a portion of the larvae from each batch are masculinized and grown on. The remainder of the larvae from each batch are grown to a size where they can be sexed visually or by histology. Fish from the groups of masculinized larvae are discarded if their untreated counterparts are of mixed sex; masculinized fish resulting from matings that gave 100% females in the progeny test are retained. The retained groups of masculinized fish are genotypic females that have a male phenotype. At maturity, the spermatozoa they produce are monosex female and can be utilized to fertilize normal ova and produce genotypically and phenotypically normal female offspring (Figure 2).

The procedure just described involves two generations of rearing except in the special case of some studies in rainbow trout where

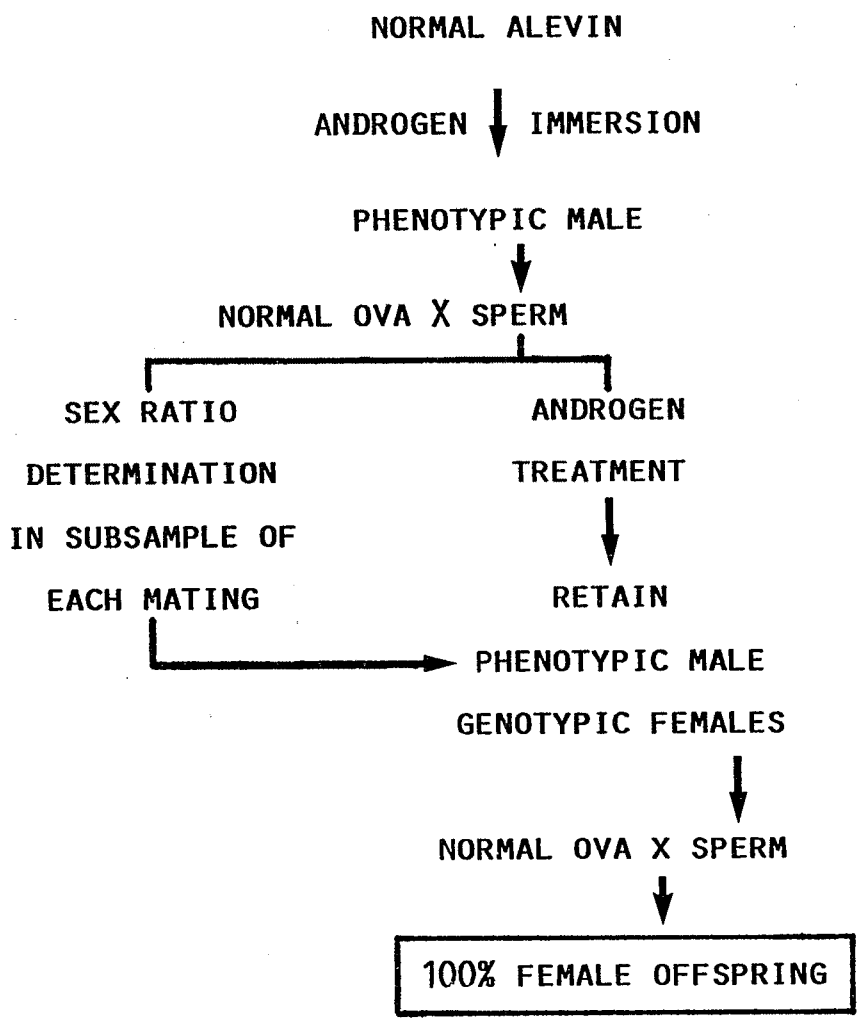

Figure 2. Production of monosex female sperm, two generation technique (reproduced with permission from Donaldson and Benfey 1987).

adult masculinized trout of mixed genotype were sorted on the basis of whether the sperm duct had developed or not. In this procedure, masculinized trout having no sperm duct were regarded as being genotypic females (Bye and Lincoln 1986).

2. Masculinization of gynogenetic larvae. This technique is being developed to obviate the need for progeny testing after masculinization thus reduce the process of producing monosex spermatozoa to one generation. In this procedure, haploid gynogenesis is induced by fertilization with UV-irradiated sperm. Diploidy then is restored by application of a temperature, pressure or chemical shock that prevents the separation of the second polar body (Thorgaard 1983, Donaldson and Benfey 1987).

   The gynogenetic embryos are reared to the stage at which sex differentiation is initiated and then masculinized by androgen treatment. The resultant fish are phenotypic male gynogens that produce monosex female spermatozoa at maturity (Figure 3). These gynogens will be less heterozygous, but the use of their spermatozoa to fertilize normal heterozygous ova should not cause a problem of inbreeding.

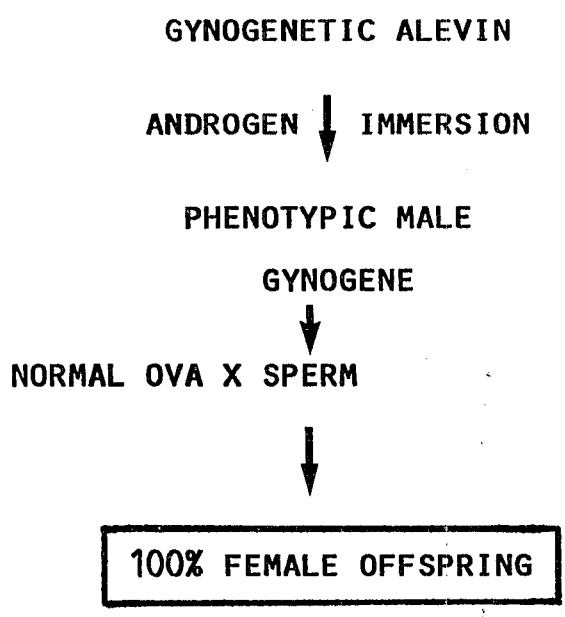

Figure 3. Production of monosex female sperm, gynogenetic technique.

PRODUCTION OF MONOSEX MALE STOCKS

DIRECT MASCULINIZATION

This procedure involves treatment of fish during early development with a natural or synthetic androgen (Figure 4). This can be achieved by either feeding, as is generally the case in tilapia culture (Guerrero 1982), or by immersion, as is used in coho and chinook salmon. In our earlier studies on Pacific salmon, we used immersion treatments of eyed eggs and alevins followed by feeding of fry with androgen treated diet. This treatment has since been reduced in chinook and coho salmon to a single 2-hour immersion treatment that is administered at or just after the time of hatching (Baker et al. 1988, Piferrer and Donaldson 1989). In the chinook study, monosex female larvae were used and it was possible to detect some masculinization after immersion in as little as 20ug/L 17α methyltestosterone (Baker et al. 1988).

EYED EGG/ALEVIN

ANDROGEN ↓ IMMERSION

PHENOTYPIC MALE

Figure 4. Direct masculinization by androgen treatment.

A potential problem in the direct masculinization procedure is the effect referred to as "paradoxical feminization" in which the proportion of females increases at high dosages of androgen (Solar et al. 1986). In recent studies using a non-aromatizable androgen, we saw no evidence of paradoxical feminization (Piferrer and Donaldson 1988).

INDIRECT PRODUCTION OF MONOSEX MALES

The generation of monosex male spermatozoa or monosex male ova for the purpose of producing all-male offspring has not been yet achieved on a production scale. For species in which the male is heterogametic, the generation of monosex male, Y chromosome bearing, sperm is more complex than the generation of monosex female sperm and involves the production of YY supermales. This can be accomplished by inducing androgenesis and using the monosex male sperm from androgenetic males to fertilize normal ova and produce normal XY all male offspring (Figure 5). The disadvantage of this technique is the technical difficulty of inducing androgenesis. In androgenesis, the egg is subjected to gamma irradiation prior to fertilization and diploidy is restored by preventing division of the first cell division (Parsons and Thorgaard 1985).

PRODUCTION OF STERILIZED STOCKS

Fish can be sterilized by a variety of techniques (Figure 6) that vary both in practicality and in level of successful sterilization achieved. The sterilization techniques that are currently being investigated involve androgen treatment with androgen, production of female triploids, and exposure to ionizing radiation. In the future, it also may be possible to produce transgenic fish in which gonadal development is blocked (N. Maclean, pers. comm.)

HORMONAL STERILIZATION

The technique of hormonal sterilization is similar to the technique for direct masculinization, except that the treatment continues for a longer period and at a higher dosage (Figure 7). In coho salmon, the alevins are treated with androgen by immersion and then by dietary administration from first feeding up to a body weight of about 2 grams. In laboratory studies, a high level of treatment success has been achieved (Goetz et al. 1979, Donaldson and Hunter 1982); but, attempts to transfer the technology to the production level have had variable success. Variations in treatment protocol in salmon can result in incomplete sterilization, a poor rate of multiplication, or in the occurrence of morphological deformities.

TRIPLOID FEMALES

Studies in salmonids (Benfey 1989) and in other species have shown that oocytes in triploid female fish do not normally undergo vitellogenic growth and that the fish retain an immature appearance. Triploid males, on the other

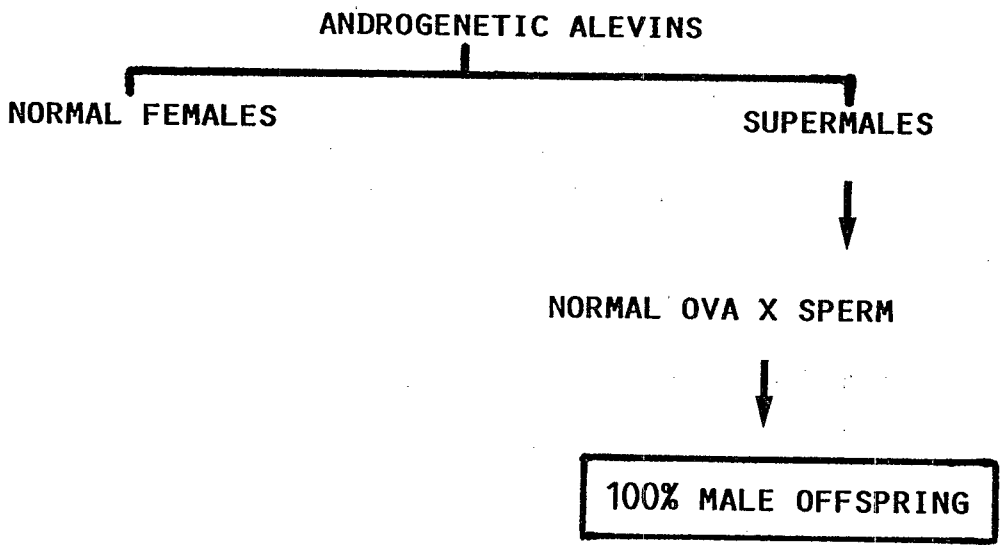

Figure 5. Production of monosex male sperm by androgenesis.

hand, do undergo testicular development, become mature in appearance, and are capable of producing aneuploid spermatozoa (Benfey et al. 1986).

Thus, to produce triploid fish that are sterile in appearance as well as in reproductive capacity, it is necessary to produce female triploids. This can be accomplished in a variety of ways (Figure 8) (Donaldson and Benfey 1987). Of the various techniques shown, the following are the most straightforward: (1) fertilization of ova with monosex female spermatozoa followed by induction of triploidy, (2) induction of triploidy in zygotes of mixed sex followed by estrogen treatment of larvae, and (3) fertilization of normal ova with diploid sperm followed by estrogen treatment of larvae. The first procedure is preferred as it will result in the production of 100% female triploids. As shown in the diagram, it should be possible, in future, to produce tetraploids that produce diploid monosex female sperm.

IONIZING RADIATION

Studies in Japan on the effects of ionizing radiation on development in fish indicated that irradiation during sex differentiation may cause selective damage to the germ cells and thus induce sterility (Egami and Ijiri 1979). Recently, sterility was induced in Atlantic salmon (Salmo salar) by gamma irradiation at the eyed embryo stage (Thorpe et al. 1987).

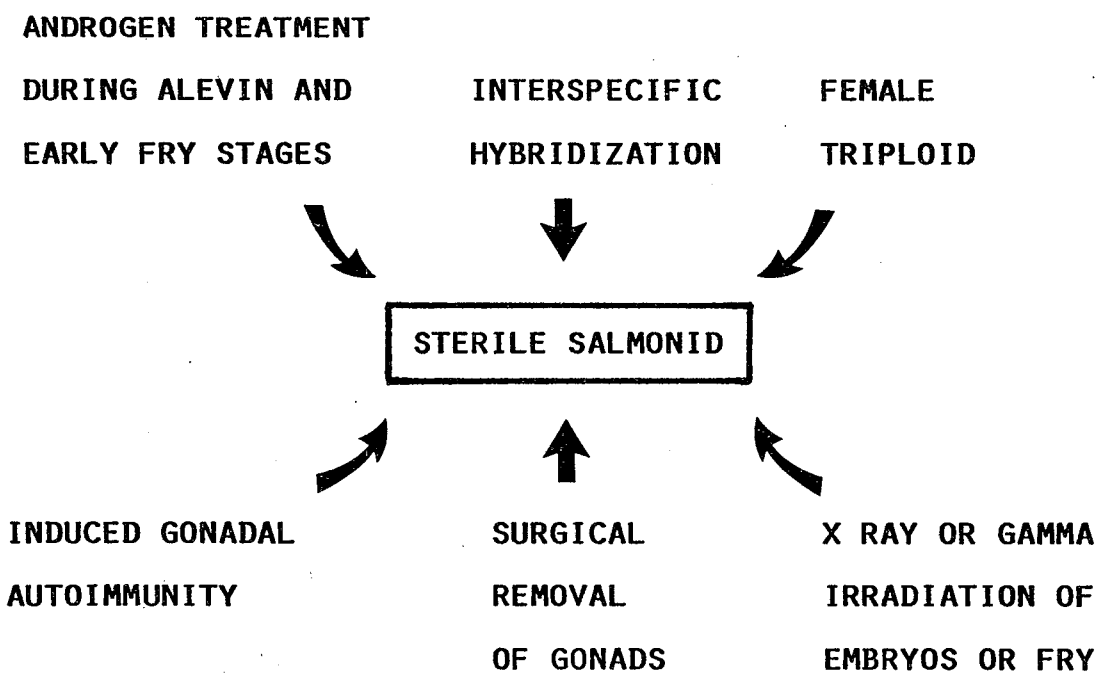

Figure 6. Sterilization techniques (reproduced with permission from Donaldson 1986).

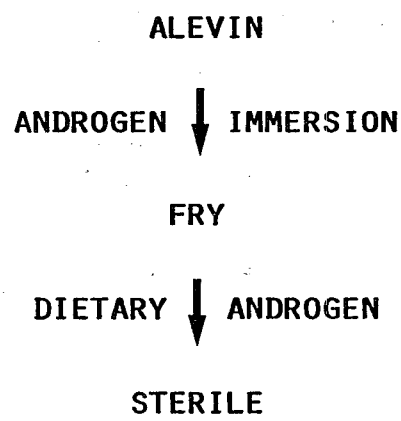

Figure 7. Hormonal sterilization using androgen treatment.

CURRENT STATUS AND FUTURE PROSPECTS

Sex control techniques currently are being applied in the culture of tilapia species, carps, rainbow trout, Atlantic salmon, coho salmon, and chinook salmon. As understanding improves of the methodologies for regulating sex in fish, we can expect to see increased application of these techniques as they promise to improve both the economics of the culture process--for example, through the rearing of all female broodstock--and the value of the fish produced--for example, by improving quality at harvest.

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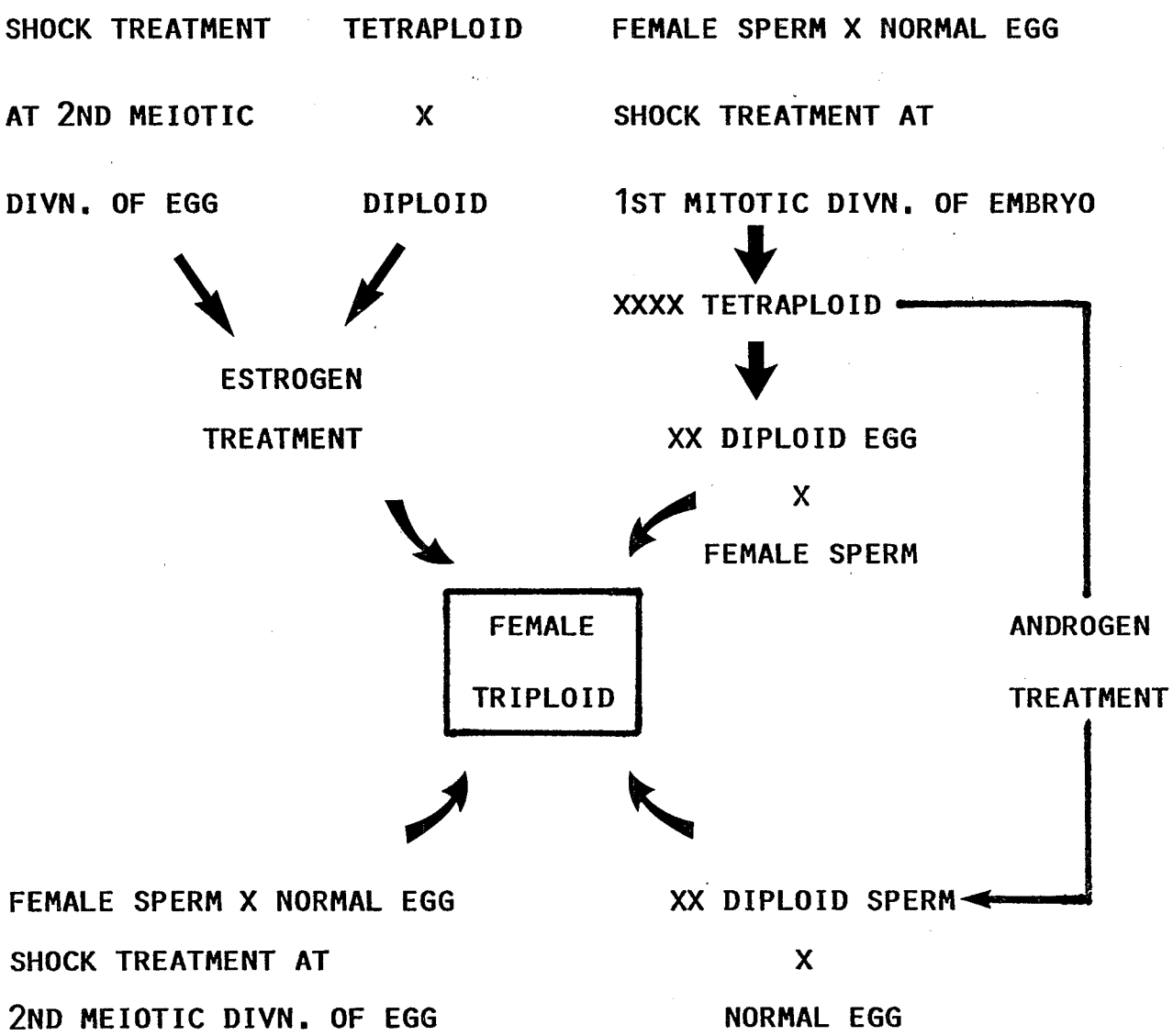

Figure 8. Sterilization by production of female triploids (reproduced with permission from Donaldson and Benfey 1987.

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ASEXUAL REPRODUCTION OF FISH

by

Chen Hongxi[1]

By means of microinjection or electric fusion, the asexual reproduction of fish involves the transfer of somatic cell nuclei to the mature unfertilized eggs or the enucleated eggs of fish of the same or different species. Just as with the zygote of the fertilized egg, the somatic cell nucleus can promote the receiving egg to cleave and develop into a normal individual. There is no fertilization, however, of the male and female pronuclear synapsis. Thus the asexual reproduction of fish is quite different not only from the normal sexual reproduction but also from the monosexual reproduction in various types. As the asexual reproduction of fish is a special and artificial method of reproduction, it can be applied widely to certain theoretical problems and to breeding practice.

THE REPRODUCTIVE MODES AND ASEXUAL REPRODUCTION OF FISH

From hermaphroditic autogamy to dioecious amphimixis, fish have many reproductive modes. Of all these modes, amphimixis is the most common phenomena. There also are a few genera, however, whose fish belong to a so-called thelytoky, producing only female progeny. Thelytoky can be divided into two types: gynogenesis and hydridogenesis. Gynogenesis is a kind of reproduction of apomixis or pseudogamy in which there is no male and female pronuclear synapsis, and only female offspring are produced with a matromorphy phenotype. It is the result of diploid parthenogenesis. On the other hand, in hybridogenesis, the male and female pronucleus will be fused with each other. The progeny are real hybrids and their phenotype is matroclinous. A few examples of parthenogenesis have occurred in Osteichthyes due to stimulations of some pathological or physicochemical factors. The thelytoky occurs depending on the sperm, but parthenogenesis does not.

Androgenesis is another kind of monosexual reproduction that occasionally appears in artificial outcross. Through artificially induced androgenesis, offspring could be produced that are both male and female. The asexual reproduction of fish described in this paper is an especially artificial reproduction, in which, as mentioned above, the fish diploid somatic nucleus is transferred to the mature unfertilized egg or enucleated egg by means of the nuclear transplantation or electric fusion. Then the egg

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will develop directly into an individual without the male and female pronuclear synapsis as happens in amphimixis. So it is neither diploid parthenogenesis nor diploid androgenesis; it is just asexual reproduction of diploid somatic nuclei. The offspring will be both male and female. Their phenotype resembles those of the nucleus donor fish. In nuclear transplantation of heterospecies, however, some characteristics of offspring may resemble those of the cytoplasm recipient fish. A comparison of the modes of reproduction in fish is provided in the Table 1.

TABLE 1. COMPARISON OF THE MODES OF REPRODUCTION IN FISH

Reproductive modes	Route of embryo development	Phenotype of offspring	Occurrence
Amphimixis	Dioecious or autogamy	Intermediate	Most common phenomena
Monosexual reproduction			
Gynogenesis	Pseudogamy	Matromorphy	Naturally or induced
Hydridogenesis	Gametogamy	Matroclinous	Naturally or "synthesis"
Androgenesis	Outcross or female nucleus inactive	Patroclinous	Occasionally or induced
Parthenogenesis	Pathological or induced	Matromorphy	Occasionally or induced
Asexual reproduction	Diploid somatic nucleus	Similar to donor fish	Artificially induced

PRESENT STATUS OF STUDIES ON FISH ASEXUAL REPRODUCTION

As stated above, fish asexual reproduction requires transfer of a somatic cell nucleus to the cytoplasm of a recipient egg by microinjection or electric fusion. The recipient egg is then promoted by the donor nucleus to cleave and develop into an individual.

NUCLEAR TRANSFER MEDIATED BY MICROINJECTION

The nuclear transplantation technique for fish was developed in China by the late professor Tong Dizhou and his colleagues. By means of nuclear transplantation, Tong Dizhou and coworkers (1980, 1984) carried out studies on the relationship of nucleus and cytoplasm, in order to explore the rule of phenotype variance and the new approach of fish breeding. They produced a hybrid fish from a carp (Cyprinus carpio) nucleus and a crucian carp (Carassius auratus) cytoplasm, and a hybrid fish from a crucian nucleus and carp cytoplasm. Both male and female of these two nuclear-cytoplasmic hybrid fish developed normally and produced F_2, F_3 and F_4 filial generations. Morphological characteristics of nuclear-cytoplasmic hybrid fish showed that some features are intermediates such as the number of lateral line scales in the combination of carp nucleus and crucian cytoplasm. Some are inherited from the nucleus donor fish, such as the barbs, and some seem to come from the cytoplasm recipient fish, such as the number of vertebrae in hydrid fish. These results indicate that both nucleus and cytoplasm can influence the expression of genetic information on the hybrid fish. The phenotype of hybrid fish result from the interaction of donor nucleus and recipient cytoplasm.

They also transplanted blastula cell nuclei of grass carp into cytoplasm of blunt anout bream (Megalobrama amblycephala) recipient eggs and obtained nine subfamiliar nuclear-cytoplasmic hybrid fish. One male individual of these hybrid fish attained sexually maturity (1985).

Taking three strains of domestic carassius goldfish (red dragon eye, red carassius, egg fish) as experimental materials, by means of nuclear transplantation technique, Wu Shangqin et al. (1980) carried out nuclear-cytoplasmic recombination among strains, in order to explore the effect of nucleus and cytoplasm to the expression of morphological characteristics and interaction between nucleus and cytoplasm. By means of four serial nuclear transplantations--i.e., transplanting the blastula cell nuclei of double tail red dragon eye goldfish into the cytoplasm of single tail red carassius recipient egg, they found increasing numbers of single tail goldfish in nuclear transplanting fish. If the single tail nuclear transplanting fish were crossed with red dragon eye, the number of single tail fish also increased. So, cytoplasm not only changes the expression of genetic characters but also can affect the second generation. This effect can be accumulated from generation to generation. The effect is still not fully understood.

Chen Hong-xi et al. (1986) transplanted subcultured cell nuclei originating from blastula cells of the crucian carp (C. auratus) into the enucleated unfertilized eggs of the same species and obtained a nuclear transplanted fish that survived for more than 3 years. They also transplanted the primary cultured cell nuclei originating from the kidney of crucian carp or goldfish into the enucleated eggs of crucian carp or goldfish and obtained mature adult fish. This result implies that the cultured cell nuclei originating from the specialized somatic cell of adult fish still can promote the recipient eggs to develop to an adult fish and retain their developmental totipotency.

Lu Renhou et al. (1982) induced the diploid chromosomes of cultured cells originating from grass carp tail fin doubling to autotetraploid cells by colchicine. Transplanting this tetraploid nuclei into loach (Misgurnus anguillicaudatus) nucleated eggs, they also obtained interfamiliar nuclear-cytoplasmic embryos, but did not obtain survival larva.

According to the Porter's method, Liu Hanqin et al. (1987) inseminated the eggs of Paramisgurnus dabryanus with the sperm of Misgurnus anguillicaudatus. In 7 to 15 minutes after artificial insemination, they mechanically removed the egg nucleus and obtained androgenetic haploid embryos reconstituted from the sperm nuclei of M. anguillicaudatus and egg cytoplasm of P. dabryanus. Then, they transplanted androgenetic haploid embryonic cell nuclei into the nucleated eggs of P. dabryanus and obtained seven androgenetic diploid adult fish. These androgenetic diploid fish were sexually mature and the androgenetic haploid embryos or fries died of "haploid syndrome." The doubling mechanism of androgenetic haploid chromosomes in the egg cytoplasm of P. dabryanus is not known yet. According to the examination of morphological characteristics, chromosomes, and isoenzymes of the androgenetic diploid fish, however, they indeed came from the androgenetic haploid with nuclear chromosomes doubling in recipient cytoplasm. So the survival rate of nuclear transplant fish obviously was enhanced.

NUCLEAR TRANSFER MEDIATED BY ELECTRIC FUSION

There have not been many reports about fish cell fusion. Tong Dizhou et al. (1973) have qualitatively proven cell fusion of goldfish blastula cells and fusion between goldfish blastula cells and Ehrlich cells mediated by Sendai virus. Mizukami et al. (1979) studied the cell fusion process in which the isolated embryonic cells of medaka (Oryzias latipes) could contact with each other again in salt solution and then were led to be fused with each other. The factors that influenced the cell fusion also were studied. Recently, many studies on fish cell fusion aiming at fish breeding by cell engineering have been made by cytogenetic research group in the Institute of Hydrobiology, Academia Sinica. Yan Kang et al. (1984) used PEG to promote cell fusion among CAB-80 cells and studied various experimental conditions carefully. They also used PEG to promote the cell fusion between blastula cells and unfertilized eggs, but did not get normal fries. Shu Xiaolin et al. (1988) used PEG to promote minisegregant nuclei to fuse with fertilized eggs in order to study the chromosome transfer mediated by minisegregant cells. She observed that the micronuclei in the cytoplasm of fertilized egg still remained in the interphase nucleus stage and did not fuse with the nucleus of the recipient egg.

Electric fusion is a new cell biological technique and is one of the most effective method in cell fusion and gene transfer. Its advantages--such as high fusion rate, simple manipulation, nontoxicity, and visualization under microscope--make it widely used in the study of cell fusion in animal, plant, and microbial cells and protoplasts. Studies on cell fusion induced by electric field in fish have not been reported yet, however. Using Chinese-made component and fittings, we have made an electric fusion device, and using this device we have studied the cell fusion between cells or between cells and eggs induced by electric field and the various factors and conditions that affected the fusion rate. The results are as follows.

Liu Peilin et al. (1988) have gotten better results with P. dabryanus blastula cell fusion. Under the conditions of 9 to 15°C, 10 to 50 microseconds of time constant and 2.5 ~ $3x10^4$ cells/ml of cell density, the biastula cells were first treated in an intermediate solution composed of 0.02 mol/L manitol and 1 mmol/L $CaCl_2$ with cells in tight contact for 2 to 5 min. Cells then were treated by electric pulse and finally were fused with each other (Table 2).

TABLE 2. EFFECTS OF FISH BLASTULA CELL FUSION INDUCED BY ELECTRIC FIELD*

	Dikaryocyte	Multikaryocyte	FI, %	Viability(%)
X±S	283±174	506±321	46.7±10.8	88.6±4.4

*Calculated from eleven experimental results.

From Table 2, we can know that, under these conditions, the fusion rate of P. dabryanus blastula cells is 46.7% and the viability is 88.6%. These results indicate that the electric fusion device is basically fit for experimental demands.

Yi Yonglan et al. (1988) used P. dabryanus, M. anguillicaudatus and red carassius as experimental materials to study the electric fusion of blastula cells and unfertilized eggs. In Table 3, it is shown that the hatching rates of fries from blastula cells fused with recipient eggs of the same species, from hybrid embryonic cells fused with P. dabryanus recipient eggs and from common carp blastula cells fused with red carassius recipient eggs are about 18.2%, 16.7% and 3.5%, respectively. In the control group, only 9 in 3778 unfertilized eggs could cleave irregularly. Most eggs cannot normally develop into the bastula stage. Under the electric pulse, the donor nucleus may enter the recipient egg and promote the egg to develop into a normal individual. Judged from outer features of electrically fused fish, it is unquestionable that the donor nucleus has participated in development of fused egg into fry. The fusion and hatching rates in the experiment are not very high, however.

Using P. dabryanus as experimental materials, Tian Jiunshi et al. (1988) studied the main conditions of electric fusion between bastula cells and unfertilized eggs and the various physicochemical and biological factors influencing the electric fusion rate. For certain electric field constants, the variety and concentration of divalent cation, the concentration of manitol, and the pretreatment condition of blastula cells have a significant influence on the fusion rate. Among effects of divalent cation, the effect of Ca^{2+} on electric fusion is the best. When the concentration of Ca^{2+} and manitol is 10^{-3} M and 0.2M, respectively, the conductance and osmotic pressure of intermediate solution are most beneficial to the fusion and hatching rates. If the donor cells are pretreated with 100 ug/ml pronase E for 6 to 10 minutes at 25°C, the fusion rate can be enhanced markedly. When the density of donor

TABLE 3. COMPARISON OF EMBRYONIC DEVELOPMENT OF RECIPIENT EGGS AFTER ELECTRIC FUSION

Donor recipient cells, eggs	Number of recipient eggs	Number of bastula stage	Number arrested in gastrula stage	Fry
P. dabryanus→ P. dabryanus	296	33	12	6 (18.2%)
P. dabryanus ( ) X M. anguillicaudatus ( ) → P. dabryanus	1107	36	10	6 (16.7%)
Cyprinus carpio → Red Carassius	662	113	18	4 (3.5%)
Blank control	2230	9		
Control	1548	0		

"→" donor cell nuclei shocked in recipient eggs using electric pulses.

cells is below 10^5 cells/ml, the direct proportion appears between fusion rate and donor cell density. But when the density is above 10^5 cells/ml, the fusion rate shows a declining trend. The quality of recipient eggs also has obvious influence on the fusion and hatching rates. A properly matured recipient egg is the basis of a successful experiment. If fusion is accomplished within 10 minutes after eggs are excited, a much higher fusion rate can be obtained. Under the optimum condition mentioned above, the fusion and hatching rates can reach 80.0% and 20%, respectively.

The cytological studies on recipient eggs indicated that most recipient eggs received 2 to 4 donor nuclei that were uncertainly located in the cytoplasm. Because every donor nucleus could develop to form a set of mitotic apparatus, several blastomeres were formed after the first cleavage. The chromosome studies on the electric fusion embryo are the same as that of donor cell (P. dabryanus X M. anguillicaudatus 2N = 74). The outer features of electric fusion fish are similar to that of donor fish.

Liu Peilin et al. (1988) improved experimental conditions further and thus enhanced the fusion and hatching rates. Using P. dabryanus as experimental material, they studied the electric fusion efficiency of blastula cells and unfertilized eggs. Normal artificial insemination eggs from the same parents were used as the control group. Here, the fertilized rate is the percentage of fertilized eggs that can develop into middle gastrula stage in all fertilized eggs. The fusion rate is the percentage of fused eggs that develop into middle gastrula stage in all recipient eggs. Thus, the fusion

efficiency is the ratio of fusion rate to the fertilized rate. The hatching efficiency of fused eggs is also the ratio of two hatching rates of experimental group to control group. The results in Table 4 are calculated from ten experimental results.

TABLE 4. ELECTRIC FUSION OF BASTULA CELLS AND UNFERTILIZED EGGS IN P. DABRYANUS

Group	Number of recipient eggs	Middle gastrula stage		Fries		Fusion efficiency (%)	Hatching efficiency (%)
		No.	(%)	No.	(%)		
Experimental group	2273	1191	52.30	191	16.04		
Control group	1420	930	65.49	354	38.06	78.86± 2.8	42.14± 2.41

From Table 4, it is seen that the electric fusion efficiency and the hatching efficiency are 79.86±2.80% and 42.14±2.41%, respectively. These are average numbers from ten experimental results and the average standard deviation is not above 5%, which indicates that the experimental conditions are favorable and the results have reached our demands.

In addition, Liu Peitin et al. (1988) studied the conditions of short-term cultured embryonic cells of P. dabryanus fused with the unfertilized eggs of the same species. Its fusion rate was 30 to 32% and 2 larval fish survived for 2 months. It seems that the electric fusion technique can cause the cultured cell to fuse with the egg too. When the nucleus enters the egg cytoplasm, the recipient egg can cleave normally and develop into an individual. The condition of electric fusion should be improved, however, in order to obtain satisfactory result on the fusion and hatching rates.

CONCLUSION

Microinjection and electric fusion are effective methods for fish asexual reproduction. The nuclear transplantation by microinjection has been studied for more than 20 years. Its advantages have been shown in the studies of nuclear-cytoplasmic relationship, the formation of nuclear-cytoplasmic hybrid, the influence of nucleus and cytoplasm on the phenotype and so on. Microinjection, however, is a complicated technique of micromanipulation. It is very difficult to avoid mechanical damage in the operation. So the survival rate of the nuclear transplantation with midblastula and cultured somatic cell are only about 15% and 1%, respectively, even for a well trained technician.

Compared with nuclear transplantation, electric fusion has many advantages--such as high fusion and hatching rates, simple manipulation, nontoxicity, simultaneous treatment of dozens of cells in electric field and less mechanical damage. On the other hand, in nuclear transfer mediated by electric fusion, the recipient eggs whose nucleus is not picked out previously always receive more than one donor cell nucleus, which increases complexity. In spite of the complexity, the fusion and hatching rates are still high. Reason for the high rates could be identified only after further investigation of the mechanism of embryo development of fused eggs. As to reasons for low hatching and survival rates of cultured somatic nuclear transplantation by microinjection or electric fusion, it also seems that, except many technical difficulties, there may be some unknown problems related to the developmental potentiality of the somatic nucleus. Only after these problems about the relation of somatic nucleus genotype and its totipotency become clearer and the dedifferentiation condition of somatic nucleus and the isolation of cell clones capable of genetic totipotency is understood, we may greatly enhance the hatching and survival rates.

The compatibility between the donor cell nucleus and the cytoplasm of recipient egg in fish is much greater than that in amphibians. According to the present data, the compatibility of intersubfamilies or intergenera can lead to producing normal individuals. That provides the favorable condition on the study about nuclear-cytoplasmic relationships and the formation of nucleus-cytoplasmic hybrids.

In fish asexual reproduction, compared with amphimixis, there are no problems such as reproductive isolation, crossing incompatibility, F_2 disjunction and hybrid sterility, etc. So, fish asexual reproduction can be widely applied in as a new approach fish breeding.

The interaction of nucleus to nucleus, nucleus to cytoplasm, and their influence on the phenotype are still not very clear. Great efforts must be made in order to solve these problems.

Although there are many difficulties in technique and theory at present, the prospects of fish asexual reproduction will be very bright. As the techniques are perfected, fish asexual reproduction will be very useful not only in theoretical research, such as nuclear-cytoplasmic relationship, the developmental potentiality of somatic nucleus and somatic cell genetics, but also in the application of somatic cell breeding, the formation of fish clones and nuclear-cytoplasmic hybrid fish and so on. Especially when the asexual reproduction technique is combined with these techniques and methods such as modern cell engineering, development engineering, gene engineering and classical breeding technique, its applied values will be unforeseen.

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INDUCED BREEDING OF CULTURED FISH IN CHINA

by

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INTRODUCTION

China has not only one of the largest freshwater areas in the world, but also a 3000-year history of fish culture. The development of fish culture in China has made rapid progress since 1949. Currently, not only ponds are used for fish culture, but also large water bodies, such as natural lakes, reservoirs, canals, small rivers and their tributaries, as well as many paddy fields. Production from these different types of water bodies is rapidly increasing; fish yields per unit in China is one of the highest in the world. Recently, China produced more than 1 million tons of fish from freshwater fish culture, which was about one-fifth of the total fisheries production, and the largest production of cultured freshwater fish in the world.

Because of the development of fish culture, more and more fry and fingerlings are needed for stocking. It is obvious, therefore, that the artificial propagation of cultivated fishes and the large scale production of fry and fingerlings are very important. Although greater production of fry and fingerlings has occurred from year to year, the demands can still not be satisfied. Because the major Chinese carp do not ovulate and spawn naturally in the confinement of ponds, nature was the only source of fry.

In 1958, under the efforts of scientists and fish farmers, successful results were obtained by the use of fish pituitary and human chorionic gonadotropin (HCG) for the induction of spawning of silver carp and bighead carp; the grass carp and the black carp, on the other hand, are not sensitive with HCG treatments, although both of them gave favourable response to fish pituitary. Since then, this technique has been spread country-wide.

HCG, however, is effective in only a few species, and the carp pituitary is very expensive. For example, it is often necessary to collect pituitary from ten or more sexually mature carp to induce spawning in a single brooder.

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Additionally, many brooder can not tolerate the injections and die after ovulation or spawning, entailing a heavy loss to fish farmers every year. Therefore, a highly effective, cheaper and safer ovulating agent was needed.

In 1974, Chinese scientists attempted to use a synthetic analogue of luteinizing hormone-releasing hormone, des-Gly^{10}(D-Ala^{6})-LHRH ethylamide (LSRH-A) as an ovulating agent (CTHAP 1977). There are many problems with the use of LHRH-A for induced ovulation and spawning of cultured fish, however, and this polypeptide apparently has had relatively little practical impact on fish farming in China. Table 1 summarizes the so-called traditional methods for induced ovulation and spawning of cultured carp in China. It is evident that LHRH-A currently is not being used alone for induced breeding of any species of cultured carp in most fish farms (Peter et al. 1987).

Under the funding of the International Development Research Centre of Canada (IDRC) and National Science Foundation of China, the proposal titled "The Basis for Application of GnRH Analogues and Dopamine Antagonists in Fish Culture" has been carried out at both the Department of Biology, Zhongshan University, Guangzhou, PRC, and Department of Zoology, University of Alberta, Edmonton, Canada, since 1984. The ultimate objective of our collaborative research program is to determine the optimal conditions for application of GnRH analogues and dopamine antagonists for induced ovulation and spawning of cultured fish.

THE LINPE METHOD--COMBINATION OF GnRH ANALOGUES AND DOPAMINE ANTAGONISTS

Our basic studies have demonstrated that the neuroendocrine regulation of GtH secretion in teleosts involves a dual control, with GtH release stimulated by gonadotropin-releasing hormone (GnRH) and inhibition of dopamine, which acts as a gonadotropin release-inhibitory factor (GRIF) on the action of GnRH as well as a spontaneous GtH release (Peter et al. 1986). The preovulatory surge of gonadotropin, which is responsible for ovulation, may be regulated by a stimulation of GnRH and a release from inhibition by GRIF.

For example, in common carp, adding males and floating vegetation as a spawning substrate to the holding pond at 6 p.m. resulted in spontaneous ovulation the following morning. A pronounced surge of GtH was found in fish that ovulated. This ovulatory gonadotropin surge lasted at least 12 hours, during which GtH levels increased about 10-fold above basal level. Serum GtH levels remained low during the course of sampling in control fish and fish that failed to ovulate (Lin et al. 1986a).

Therefore, the lack of an effect of LHRH-A alone on ovulation in carp resulted from a failure to stimulate an increase in serum GtH levels of similar magnitude to that found in fish that ovulate spontaneously. This is a result of the predominant influence of dopamine inhibition on gonadotropin secretion.

Figure 1 presents a general model of the neuroendocrine regulation of GtH secretion in teleosts. The levels of intervention and action of several catecholaminergic drugs on GtH release are indicated.

TABLE 1. SOME TRADITIONAL METHODS OF INDUCED SPAWNING OF CULTURED FRESHWATER FISH IN CHINA (adapted from Peter et al. 1988)[a]

Water Species	Temp.	First injection	Interval	Second injection	Time to ovulation (or spawning) following last injection
Common carp	20-30°C	CPE[b] (2-4/kg)	---	---	14-12 hr
		hCG[c] (800-1000 IU/kg)	---	---	14-12 hr
Silver carp	20-30°C	CPE (2-4/kg)	5-6 hr	CPE (10-20/kg)	8-6 hr
		hCG (100-200 IU/kg)	5-6 hr	hCG (700-1000 IU/kg)	8-6 hr
		hCG (100-200 IU/kg)	5-6 hr	hCG (400 IU) + LHRH-A (10 μg/kg)	8-6 hr
Mud carp	22-28°C	CPE (2-4/kg)	4-5 hr	CPE (16-30/kg)	6-4 hr
		CPE (2/fish) + LHRH-A (100 μg/kg)	4-5 hr	CPE (10-16/kg) + LHRH-A (100 μg/kg)	6-4 hr
Bream	22-30°C	CPE (2-4/kg)	5-6 hr	CPE (12-24/kg)	10-8 hr
		CPE (2/fish) + LHRH-A (10 μg/kg)	5-6 hr	CPE (6-10/kg) + LHRH-A (50 μg/kg)	10-8 hr
Grass carp	18-30°C	CPE (1-2/kg)	5-6 hr	CPE (6-12/kg)	8-6 hr
		CPE (1/kg)	5-6 hr	CPE (4-10/kg) + LHRH-A (10 μg/kg)	8-6 hr
		LHRH-A (10 μg/fish)	5-6 hr	CPE (2-4/kg) + LHRH-A (10 μg/kg)	8-6 hr

TABLE 1. (continued)

Species	Water Temp.	First injection	Interval	Second injection	Time to Ovulation) or spawning) following last injection
Bighead carp	20-30°C	CPE (2-4/kg)	5-6 hr	CPE (10-20/kg)	8-6 hr
		hCG (100-200 IU/kg)	5-6 hr	hCG (700-1000 IU/kg)	8-6 hr
		hCG (100-200 IU/kg)	5-6 hr	hCG (400 IU/kg) + LHRH-A (10 μg/kg)	8-6 hr
Black carp	20-30°C	CPE (2-4/kg)	6-8 hr	CPE (16-30/kg)	10-8 hr
		CPE (2/kg) + LHRH-A (100 μg/kg)	4-5 hr	CPE (10-16/kg) + LHRH-A (100 μg/kg)	10-8 hr

[a]Information gathered from a number of fish hatcheries in Guangdong Province, PRC.

[b]CPE = carp pituitary extract.

[c]hCG = human chorionic gonadotropin.

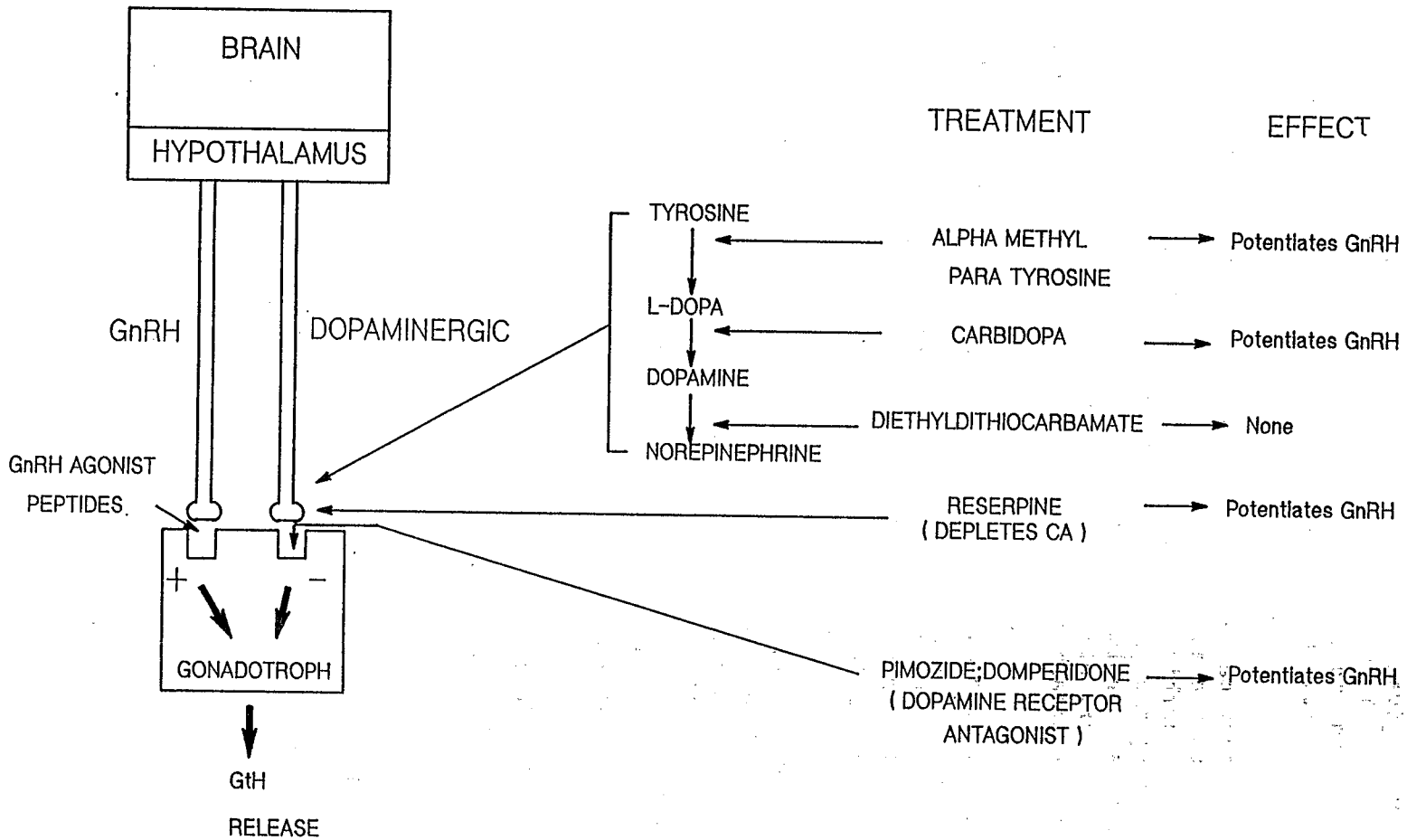

Figure 1. Model of neuroendocrine regulation of GtH release in teleosts (from Peter et al. 1987a).

Laboratory experiments on goldfish were the first to reveal the effect of blockage of dopamine actions on GtH release and ovulation by determining the response to LHRH-A, when given in combination with the dopamine receptor antagonist pimozide(PIM). In these studies, combined injections of PIM and LHRH-A resulted in a rapid increase in the blood level of GtH and ovulation within a predictable time following injection (Chang and Peter 1983; Peter et al. 1986, 1987).

Reserpine, a drug that causes general depletion of catecholamine, also significantly potentiates the action of GnRH and its analogues. The drug α-methyl-para-tyrosine and carbidopa, which blocks catecholamine synthesis at steps up to and including the production of dopamine, potentiates the actions of LHRH-A on GtH release in goldfish (Peter et al. 1986) and Chinese loach (Lin et al. 1986b); however, blocking conversion of dopamine to norepinephrine with the drug diethyldithiocarbamate has no apparent effect on the actions of LHRH-A.

Results from experiments done on a number of species of cultured Chinese carp (Lin et al. 1986a, 1987a,b; Peter et al. 1986, 1987, 1988) all confirm that dopamine acts as GRIF and that dopamine receptor antagonists or drugs that block dopamine synthesis potentiate the activity of GnRH analogues. This combined treatment is highly effective in stimulating GtH secretion and ovulation.

To maximize the efficiency of this combined treatment for induced ovulation, research was done to determine the most effective GnRH analog and dopamine antagonist to use for induced ovulation of cultured fish. Structure-activity studies on gonadotropin-releasing hormone in the goldfish indicate that an analogue of salmon gonadotropin-releasing hormone, (D-Arg^6, Trp^7, Leu^8, Pro^{9}NEt)-LHRH(sGnRH-A), is the most active peptide of all those tested (Peter 1986; Peter et al. 1985).

In the presence of PIM, sGnRH-A is 17 times more potent than LHRH-A in stimulating GtH secretion in goldfish (Peter et al. 1985). In the presence of a dopamine antagonist, sGnRH-A also was more effective than LHRH-A in stimulating GtH secretion and ovulation in Chinese loach and common carp (Lin et al. 1988). The activity of catecholaminergic drugs in potentiating the activity of LHRH-A was investigated in goldfish (Peter et al. 1986) and Chinese loach (Lin et al. 1986b). Many of these drugs, including a number of dopamine receptor antagonists that are specific for D-2 type receptors, potentiate the actions of GnRH analogues. Domperidone (DOM), a specific D-2 antagonist that does not cross the blood-brain barrier, is highly potent in potentiating the actions of LHRH-A and sGnRH-A (Peter et al. 1986, Omeljaniuk et al. 1987, Lin et al. 1988).

For example, common carp, when receiving the combination of sGnRH-A and DOM, experience a very rapid increase in serum GtH level at 6 to 24 hours after injection, similar to a spontaneous ovulatory surge of gonadotropin. This combination treatment is highly effective in inducing ovulation within 14 hours following injection (at 21-23°C) (Lin et al. 1987b).

Experiments have been done on grass carp (Ctenopharyngodon idellus), silver carp (Hypophthalmichthys molitrix), bighead carp (Aristichthys nobilis), black carp (Mylopharyngodon piceus), mud carp (Cirrhinus molitorella), and bream (Parabramis pekinensis) as well as some other cultured fish in China, demonstrating that injection of the combination of dopamine antagonist (PIM or DOM) plus GnRH analogues (LHRH-A or sGnRH-A) is highly effective in inducing ovulation and spawning (Lin et al. 1985, 1986a,b, 1987a,b; Peter et al. 1987, 1988). The effectiveness of this new technique, called the Linpe method, for induced ovulation of cultured fish was judged against several criteria. The criteria are a high rate of ovulation that occurs consistently from one group of brooders to another within each species, a series of ovulations that are complete rather than partial, a short and predictable time-to-ovulation following injection, a consistent production of fertile and viable ovulated eggs, and a no-effect consequence for subsequent reproductive cycles by the same brood fish (Peter et al. 1988). The results of the experiments on each species of cultured fish were highly successful in meeting these criteria (Peter et al. 1987, 1988). On the basis of these results, the optimal treatments in common carp, silver carp, mud carp, grass carp, bream, bighead carp, black carp, loach, Thailand mud carp (Labeo rohita), Chinese catfish (Clarias fuscus) and African catfish (Clarias gariepinus) are summarized in Table 2.

RESULTS OF FIELD TRIALS

In cooperation with several fish farms and fish hatcheries in Guangdong Province, field trials were done on a number of species to test the Linpe method. The results of the field trials demonstrate that the combination of DOM plus LHRH-A or sGnRH-A is highly effective for induced ovulation or spawning of silver carp, mud carp, grass carp, bighead carp, black carp and Thailand carp (Peter et al. 1988). First and second spawning in the same reproductive season have been induced with DOM and LHRH-A in silver carp, grass carp, bighead carp, and mud carp, and in successive reproductive seasons with silver carp, grass carp and mud carp. The results indicate that successive reproductive cycles are unaltered by induced ovulation and spawning with the Linpe method.

The results of field trials in 1986-1987 have provided an adequate basis for commercialization of the Linpe method for induced ovulation and spawning of the cultured freshwater fish species worked on, as well as on closely related species. With the help of IDRC, an adequate supply of 3 kg DOM has been acquired for marketing and expanded field trials for approximately 600,000 to 800,000 kg of brood stock fish (based on our recommended dosages of DOM). A prototype spawning kit, using this DOM and LHRH-A made by Ningbo Fish Hormone Factory, was marketed to the key fish hatcheries in 1988. In the Delta Area of Pearl River, Guangdong Province, more than 500 g DOM has been utilized for induced spawning of about 125,000 kg of brood stock fish, producing approximately 6,250 million fry. For example, the Nanhai Fish Hatchery, one of the largest in China, completely adopted the Linpe method instead of carp pituitary injection for inducing spawning of grass carp and mud carp in 1988. Hatchery personnel administered 80 g of DOM and suitable amounts of LHRH-A, induced 1266 brooders of grass carp (average body weight,

TABLE 2. LINPE METHOD OF INDUCED OVULATION AND SPAWNING OF CULTURED FRESHWATER FISH IN CHINA

Water Species	Temp.	First injection	Interval	Second injection	Time to ovulation (or spawning) following last injection
Common carp	20-25°C	domperidone (5 mg/kg) + LHRH-A (10μg/kg)	---	---	16-14 hr
		domperidone (1 mg/kg) + sGnRH-A (10 μg/kg)	---	---	16-14 hr
Silver carp	20-30°C	domperidone (5 mg/kg) + LHRH-A (20 μg/kg)	---	---	12-8 hr
		domperidone (5 mg/kg) + sGnRH-A (10 μg/kg)	---	---	12-8 hr
Mud carp	22-28°C	domperidone (5 mg/kg) + LHRH-A (20 μg/kg)	---	---	6 hr
Bream	22-30°C	domperidone (3 mg/kg) + LHRH-A (10 μg/kg)	---	---	10-8 hr
Grass carp	18-30°C	domperidone (5 mg/kg) + LHRH-A (10 μg/kg)	---	---	12-8 hr
Bighead	20-30°C	domperidone (5 mg/kg) + LHRH-A (50 μg/kg)	---	---	12-8 hr
Black carp	20-30°C	domperidone (3 mg/kg) + LHRH-A (10 μg/kg)	6 hr	domperidone (7 mg/kg) + LHRH-A (15 μg/kg)	6-8 hr
Loach	18-30°C	domperidone (1 mg/kg) + LHRH-A (50 μg/kg)	---	---	14-11 hr

TABLE 2. (continued)

Water Species	Temp.	First injection	Inter-val	Second injection	Time to ovulation (or spawning) following last injection
		domperidone (5 mg/kg) + sGnRH-A (1 μg/kg)	---	---	14-11 hr
Thailand mud carp	22-30°C	domperidone (3.5-5mg/kg) +LHRH-A (20-50μg/kg)	---	---	6 hr
Chinese catfish	22-30°C	domperidone (10 mg/kg) +LHRH-A (50-100μg/kg)	---	---	18-16 hr
African catfish	22-30°C	domperidone (5 mg/kg) +LHRH-A (50 μg/kg)	---	---	10-8 hr

10 kg) and more than 1500 brooders of mud carp (average body weight, 1 kg) spawning, with a rate of spawning of 90%, a rate of fertilization of 80%, a rate of hatching of 90%, and the production of about 750 millon fry.

Thus, the Linpe method is now a consistently highly effective and reliable technique that can be widely applicable to induce ovulation and spawning of cultured fish. The species tested include common carp (Cyprinus carpio), silver carp (Hypophthalmichthys molitrix), bighead carp (Aristichthys nobilis), black carp (Mylopharyngodon piceus), grass carp (Ctenopharyngodon idellus), mud carp (Cirrhinus molitorella), Thailand mud carp (Labeo rohita), bream (Parabramis pekinensis), loach (Paramisgurnus dabryanus), Chinese catfish (Clarias fuscus), African catfish (Clarias gariepinus), pacu (Clossoma brachypomum), mandarinfish (Siniperca chuatsi), and silvery chub (Xenocypris argentea).

SUMMARY

In summary, it is clear that the GRIF action of dopamine is found in a wide phylogenetic range, and perhaps in all teleosts. In many teleosts, the endogenous GRIF activity of dopamine can effectively modulate the GtH-releasing activity of exogenous GnRH to the extent that a high rate of ovulation cannot be induced by injection of superactive GnRH agonist alone. The use of DOM, a highly potent dopamine receptor antagonist, in combination with GnRH analogues, such as sGnRH-A, represents a highly effective means of inducing ovulation and spawning in a number of cultured freshwater fish.

The main advantages of the Linpe method compared to the traditional methods include reduced cost of the synthetic drugs, long stability of the drugs, high predictability of the time from injection to ovulation, decreased stress on broodstock due to the necessity for only a single set of injections, and lack of any side-effects on the reproductive cycle or immune system. The potential commercialization of the Linpe method for induced ovulation and/or spawning of cultured freshwater fish will benefit the development of aquacultural production. Based on the results of our studies, a spawning kit containing sGnRH-A and DOM will soon be marketed by Syndel Laboratories, Canada, and Ningbo Fish Hormone Factory, China.

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ROLE OF STEROID HORMONES IN GONADAL GROWTH AND MATURATION IN TELEOSTS

by

Yoshitaka Nagahama

INTRODUCTION

It is well established that gonadotrophic hormones play important roles in the physiological regulation of gonadal function in teleosts. In most cases, however, gonadotropin action on gonadal development is not direct, but rather, occurs through the biosynthesis of gonadal steroid hormones that, in turn, mediate various stages of gametogenesis including oocyte growth, oocyte maturation, spermatogenesis, and spermiation. This article reviews some of our findings, mainly based on the amago salmon, Oncorhynchus rhodurus, on the physiological regulation of gonadal function by steroid hormones and the sites and mechanisms of gonadal steroid biosynthesis.

STEROIDAL MEDIATORS OF OOCYTE GROWTH AND MATURATION

Vertebrate oocytes grow while arrested in the first meiotic prophase. In teleosts, as in other nonmammalian vertebrates, the principal events responsible for the enormous growth of the oocyte involve the sequestration and packaging of a hepatically derived plasma precursor, vitellogenin, into yolk protein (Wallace 1985). It seems most likely that vitellogenesis is promoted by a two-step mechanisms in which gonadotropins increase ovarian secretion of estradiol-17β, which in turn stimulates the hepatic synthesis and secretion of vitellogenin (Figure 1). Vitellogenin then is selectively taken up from the blood stream by developing oocytes. In all of the teleost species studied so far, elevated levels of estradiol-17β have been reported in females during active vitellogenesis (Figure 2).

Besides stimulating estradiol-17β production, gonadotropin is thought to stimulate the ovary to incorporate vitellogenin into the oocytes; however, the details of this action of gonadotropins are unknown. There is now biochemical evidence that teleosts, similar to other vertebrates, probably possess two gonadotropins (Idler and Ng 1983, Suzuki et al. 1988). In my review, the term gonadotropin is taken to refer to the glycoprotein-rich "maturational" gonadotropin (Idler and Ng 1983) or GTH II (Suzuki et al. 1988).

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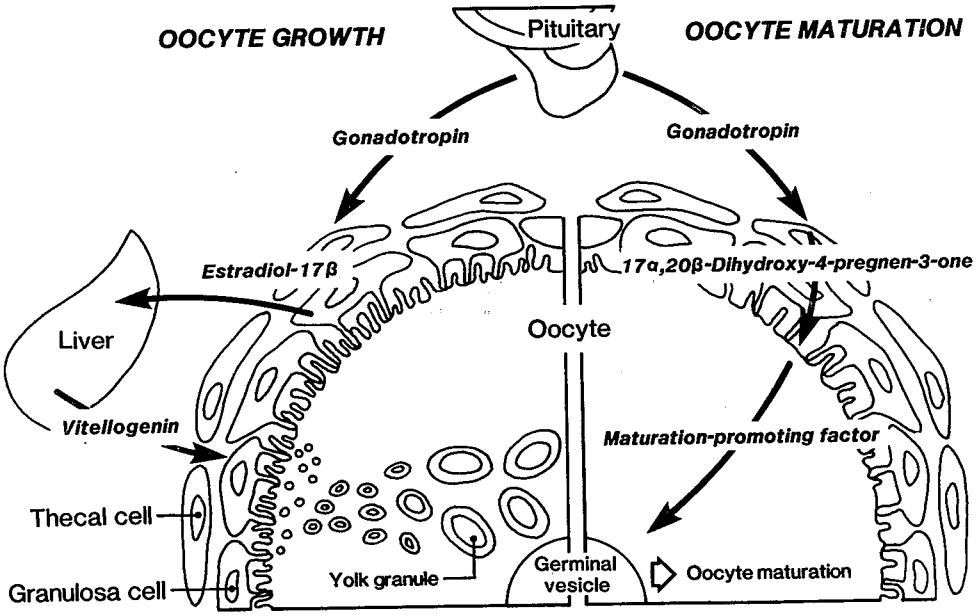

Figure 1. Hormonal control of oocyte growth and maturation in salmonids.

A period of oocyte growth is followed by a process called oocyte maturation (resumption of meiosis), which is accompanied by several maturational processes in the nucleus and cytoplasm of the oocyte. These changes occur prior to ovulation and are prerequisite for successful fertilization; they consist of the breakdown of the germinal vesicle (GVBD), chromosome condensation and extrusion of the first polar body (Masui and Clarke 1979, Goetz 1983, Nagahama 1987c).

In vitro systems have proven useful for investigating the mechanisms of oocyte maturation in teleosts. It has been demonstrated that gonadotrophic hormones stimulate meiotic maturation in follicle-enclosed oocytes, but not in defolliculated oocytes (Goetz 1983, Nagahama 1987c). Cyanoketone, a specific inhibitor of 3β-hydroxy-Δ^5-steroid dehydrogenase, completely abolished the maturational effects of gonadotropin (Young et al. 1982). These findings suggested that gonadotropins may trigger maturation by stimulating the follicle cells to synthesize a Δ^4 steroid that acts directly on the oocyte to cause maturation (Figure 1).

A variety of C21-steroids have been shown to induce oocyte maturation in vitro (Jalabert 1976, Iwamatsu 1978, Fostier et al. 1983, Goetz 1983, Nagahama et al. 1983, Greeley et al. 1986, Canario and Scott 1988, Trant and Thomas 1988). These include progesterone, 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-diOHprog), 17α,20β,21-trihydroxy-4-pregnen-3-one, cortisol, and deoxycorticosterone. Testosterone as well as other C19-steroids have been shown to induce oocyte maturation only at high concentrations. Estradiol-17β

and other C18-steroids generally are not effective in including oocyte maturation in teleost oocytes.

Recently we purified and characterized, for the first time in any vertebrate, the maturation-including hormone of amago salmon from media in which immature but fully grown folliculated oocytes of amago salmon had been incubated with partially purified chum salmon, Oncorhynchus keta, gonadotropin (SGA, Syndel Lab., Canada) (Nagahama and Adachi 1985). Twenty separate fractions were obtained by the use of reversed-phase high performance liquid chromatography. Maturation-inducing activity was found only in one fraction that had a retention time coinciding exactly with 17α,20β-diOHprog. The purity and final characterization of this active fraction were confirmed further by thin layer chromatography and mass spectrometry with authentic 17α,20β-diOHprog.

A specific radioimmunoassay for 17α,20β-diOHprog developed in our laboratory has been applied to measure blood concentrations of this steroid during the sexual maturation of female amago salmon. 17α,20β-DiOhprog levels were low in vitellogenic females. A dramatic increase occurred in mature and ovulated females (Young et al. 1983b). We also found that in masu salmon,

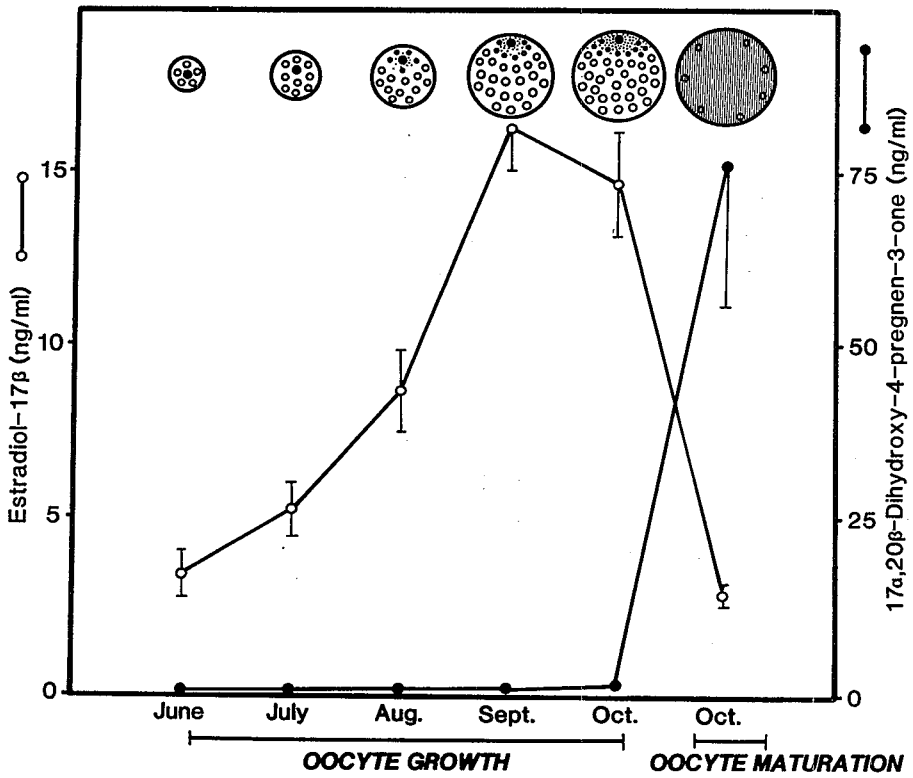

Figure 2. Changes in plasma estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one levels during sexual maturation of female amago salmon.

Oncorhynchus masou, plasma 17α,20β-diOHprog levels peaked 2 to 4 days prior to ovulation, coincident with the occurrence of GVBD of oocytes (Yamauchi et al. 1984). The relative effectiveness of a range of pregnene derivatives in inducing GVBD was investigated in vitro using amago salmon oocytes. Of the steroids tested, 17α,20β-diOHprog was found to be the most effective inducer of GVBD (Nagahama et al. 1983). Taken together, these results indicate that 17α,20β-diOHprog is the major naturally occurring maturation-inducing hormone in amago salmon (Nagahama 1987b).

Further studies from our laboratory and others suggest that 17α,20β-diOHprog functions as the maturation-inducing hormone common to several salmonids (Fostier et al 1973, Jalabert 1976, Goetz 1983, Scott et al. 1983, Canario and Scott 1988). It also is possible that 17α,20β-diOHprog acts as an important steroidal mediator of oocyte maturation in some nonsalmonid teleosts (Goetz 1983, Stacey et al. 1983, Scott et al. 1984, Greeley et al. 1986, Upadhyaya and Haider 1986). Recently, 17α,20β,21-trihydroxy-4-pregnen-3-one has been identified as a naturally occurring maturation-inducing steroid of Atlantic croaker, Micropogonias undulatus (Trant et al. 1986, Trant and Thomas 1988). 17α,20β,21-Trihydroxy-4-pregnen-3-one does not appear to be involved in the induction of oocyte maturation in salmonids, however, because there is no evidence for the presence of large amounts of this steroid in the blood of female salmonids undergoing maturation or ovulation (Canario and Scott 1988).

The site of 17α,20β-diOHprog action in inducing oocyte maturation in teleosts is considered to be at or near the oocyte surface (Nagahama 1987c) (Figure 1). 17α,20β-DiOHprog was found to be ineffective in inducing oocyte maturation when microinjected into fully grown immature oocytes of the goldfish, Carassius auratus, but was effective when applied externally. These results suggest that 17α,20β-diOHprog acts indirectly on germinal vesicles to induce resumption of meiosis. Previous studies of meiotic maturation in amphibian oocytes have revealed that cytoplasm from maturing oocytes induces GVBD when injected into immature oocytes (Masui and Clarke 1979). The activity responsible for causing GVBD is called "maturation-promoting factor" or MPF.

It, therefore, was anticipated that a cytoplasmic factor similar to amphibian MPF is newly produced in fish oocytes under the influence of 17α,20β-diOHprog. The hyaline layer obtained from goldfish oocytes treated with 17α,20β-diOHprog was able to induce meiotic maturation when injected into immature recipient oocytes (Nagahama and Yamashita 1988). Thus, MPF activity was present in the cytoplasm of 17α,20β-diOHprog-treated goldfish oocytes undergoing GVBD. Furthermore, MPF from mature oocytes of goldfish-induced GVBD when injected into the South African clawed toad, Xenopus laevis, oocytes and vice versa. It also was shown previously that goldfish MPF induced maturation of immature oocytes of the starfish, Asterina pectinifera (Kishimoto 1988). These results suggest that MPF is similar among vertebrates and invertebrates. Moreover, MPF has been detected during metaphase in mitotic cells of many species, ranging from yeast to man. Thus, MPF may be a widespread initiator of metaphase in both meiotic and mitotic cells (Kishimoto 1988).

STEROIDOGENESIS IN THE OVARIAN FOLLICLE: TWO-CELL TYPE MODEL

Ovarian follicles of teleosts, like those of other vertebrates, are composed of two major cell layers, an outer thecal cell layer and an inner granulosa cell layer (Nagahama 1983) (Figure 1). Development of a simple dissection technique to separate the ovarian follicles of salmonids into two cell layers, the thecal and granulosa layers, has made it possible to elucidate the relative contributions of each layer and gonadotropin in the overall process of estradiol-17β and 17α,20β-diOHprog. Our in vitro studies indicate that the thecal and granulosa cell layers cooperate in the production of these two steroidal mediators of oocyte growth and maturation.

ESTRADIOL-17β PRODUCTION DURING OOCYTE GROWTH

In amago salmon, estradiol-17β levels in the plasma increase during oocyte growth and rapidly decline prior to oocyte maturation (Kagawa et al. 1983). The capacity of intact follicles to produce estradiol-17β in response to gonadotropin stimulation increases during oocyte growth, but rapidly decreases in association with the ability of the oocyte to mature in response to gonadotropin (Kagawa et al. 1983).

Using various follicular preparations obtained from vitellogenic amago salmon, we examined the effects of purified salmon gonadotropin on estradiol-17β production. Gonadotropin stimulates estradiol-17β production by intact follicles and thecal and granulosa cell layer co-culture preparations, but not by the isolated thecal and granulosa cell layers (Kagawa et al. 1982). These results indicate that both cell layers are necessary for gonadotropin-stimulated estradiol-17β production. In contrast, gonadotropins greatly stimulated testosterone production by thecal layers but only slightly stimulated testosterone production by the other follicular preparations. Incubation of granulosa layers with exogenous testosterone resulted in elevated estradiol levels, whereas isolated thecal layers incubated with testosterone produced relatively small amounts of estradiol-17β, which should be attributed to contamination of thecal layer preparations with granulosa cells. Experiments examining the effects of conditioned media from incubates of one follicle cell layer on steroidogenesis by the other cell layer revealed that the thecal cell layer produced a steroid precursor that was metabolized by estradiol-17β in the granulosa cell layer. We further identified testosterone as the major aromatizable androgen produced by thecal cell layers in response to gonadotropin (Adachi and Nagahama, unpublished).

In consideration of all of these data, a two-cell type model in the production of follicular estradiol-17β has been proposed (Figure 3). In this model, the thecal cell layer, under the influence of gonadotropin, secretes the androgen substrate (testosterone), which diffuses into the granulosa cell layer where the aromatase is localized exclusively (Kagawa et al. 1985). Estradiol-17β then would have to be transported back to the thecal layer for secretion into the blood stream, because the granulosa layer is devoid of capillaries. In a recent study, we found that the thecal cell layer from amago salmon and the granulosa cell layer from the rainbow trout, Salmo gairdneri, could produce the same effect as has been reported using combination of thecal and granulosa cell layers form the same species. The

reciprocal use of amago salmon granulosa and rainbow trout thecal cell layers also is effective. This finding implies that there may be little species specificity of each of these cell layers among salmonids (Nagahama 1987b).

In Vitro production of testosterone by the isolated thecal layer preparations obtained each month during oocyte growth and maturation has revealed that the capacity of the thecal layer to produce testosterone in response to gonadotropin gradually increased during the course of oocyte growth and peaked during the post-vitellogenic period; this capacity of thecal cell layers was maintained by the period oocyte maturation and ovulation (Kanamori et al. 1988). As identical seasonal pattern of stimulation of testosterone production was observed when thecal cell layers were incubated with forskolin and agents known to raise intracellular cAMP (Kanamori and Nagahama 1988b). Furthermore, specific gonadotropin receptors were demonstrated in both thecal and granulosa cell layers from vitellogenic follicles (Kanamori and Nagahama 1988a). Therefore, these results are consistent with the general hypothesis that gonadotropin acts on thecal cell layers to stimulate testosterone production through a receptor-mediated adenylate cyclase-cAMP system.

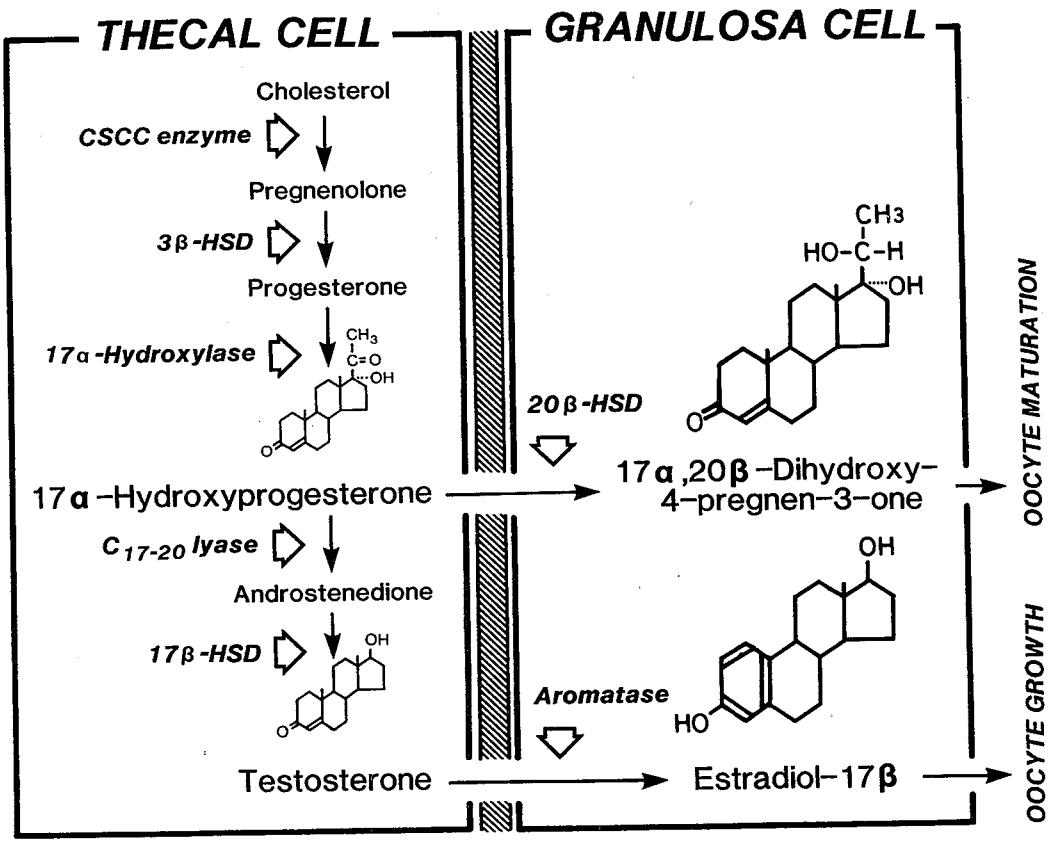

Figure 3. Two-cell type model for the production of estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one by salmonid ovarian follicles.

Aromatase activity is granulosa cell layers increased during vitellogenesis and decreased rapidly in association with the ability of the oocyte to mature in response to gonadotropin (Young et al. 1983a, Kanamori et al. 1988). This decrease in aromatase activity appears to be coincident with the decreased ability of intact follicles to produce estradiol-17β in response to gonadotropin. Because testosterone production in thecal cell layers did not decline during this time, the reduced production of estradiol-17β by postvitellogenic follicles is due, in part, to decreased aromatase activity in granulosa cell layers. Although the mechanism of the induction or activation of the salmonid granulosa cell aromatase system is unknown at this point, we recently have found that in the medaka, Oryzias latipes, which has a reproductive cycle much shorter than that of salmonids, gonadotropin stimulates the de novo synthesis of aromatase via an adenylate cyclase-cAMP dependent step (Matsuhisa et al., unpublished). It is possible that prolonged treatment of salmonid granulosa cell layers with gonadotropin may stimulate aromatase activity.

17α,20β-DiOHprog PRODUCTION DURING OOCYTE MATURATION

17α,20β-DiOHprog production by intact follicles at different stages of development showed that the capacity of the follicles to respond to gonadotropin by synthesizing this steroid was acquired immediately prior to the natural maturation period (Young et al. 1983b, Kanamori et al. 1988). Using incubation techniques similar to those used for the studies on follicular estradiol-17β production, a two-cell type model has been proposed, for the first time in any vertebrate, for the follicular production of maturation-inducing hormone (Figure 3). In this model, the thecal cell layer produces 17β-hydroxyprogesterone that traverses the basal lamina and is converted to 17α,20β-diOHprog by the granulosa cell layer where gonadotropin acts to enhance the activity of 20β-hydroxysteroid dehydrogenase (20β-HSD), the key enzyme involved in the conversion of 17β-hydroxyprogesterone to 17α,20β-diOHprog (Young et al. 1986, Nagahama 1987a,b).

The first step of the stimulating effect of gonadotropin in thecal cell layers is receptor-mediated activation of adenylate cyclase and formation of cAMP, the major site of action probably occurring at the steroidogenic step between cholesterol and pregnenolone (cholesterol side-chain cleavage enzyme systems (Kanamori and Nagahama 1988a,b). Gonadotropin appears to promote formation of one or more labile proteins required for the delivery of cholesterol to the mitochondrial cytochrome P-450 system in thecal cell layers. In contrast, granulosa cells lack the side-chain cleavage enzyme systems. The action of gonadotropin on 20β-HSD enhancement in granulosa cells was mimicked by forskolin, by dbcAMP, but not dbcGMP, and by two phosphodiesterase inhibitors (Nagahama et al. 1985a, Kanamori and Nagahama 1988b). Furthermore, gonadotropin and forskolin caused a rapid accumulation of cAMP with maximum levels at 30 to 60 min. These findings are consistent with the view that cAMP is the second messenger of gonadotropin action.

In addition to cAMP, calcium appears to play an important role in the gonadotropin regulation of steroidogenesis in amago salmon granulosa cells. Calmodulin inhibitors such as trifluoroperazine (TFP), N-(6-aminohexyl)-1-naphthalenesulfonamide hydrochloride (W5) and N-(6-aminohexyl)-5-chloro-1-

naphthalenesulfonamide (W7) strongly prevented gonadotropin, cAMP, and forskolin-stimulated 20β-HSD activation in granulosa cells in a dose-dependent manner (Nagahama, unpublished).

In vitro experiments utilizing both protein synthesis inhibitors (cycloheximide and puromycin) and RNA synthesis inhibitors (actinomycin D, cordycepin and α-amanitin) have revealed that the gonadotropin and cAMP induction of 20β-HSD activity in graulosa cell layers is dependent on the activation of new RNA and protein synthesis. Our time course studies further suggest that de nove synthesis of 20β-HSD in vitro in response to gonadotropin and dbcAMP occurs, and consists of gene transcriptional events within the first 6 hours of exposure to gonadotropin and dbcAMP and transcriptional events 6 to 9 hours after the exposure to gonadotropin and cAMP (Nagahama et al. 1985b). Thus, these results suggest that gonadotropin causes the de novo synthesis of 20β-HSD in the amago salmon granulosa cell through a mechanism dependent on RNA synthesis. The induction of 20β-HSD activity by gonadotropin in amago salmon granulosa cells is a good example of differentiated functions expressed by the target cells in response to peptide hormone stimulation.

Immediately prior to oocyte maturation, intact follicles acquire an increased ability to produce 17α,20β-diOHprog in response to gonadotropin. Although granulosa cell layers first acquired the ability to convert exogenous 17α-hydroxyprogesterone to 17α,20β-diOHprog in response to gonadotropin about 2 months prior to oocyte maturation, thecal cell layers did not develop the ability to produce 17α,20β-diOHprog in response to gonadotropin until immediately prior to oocyte maturation. Thus, thecal cell function seems to be especially critical for the acquisition of intact follicles to produce 17α,20β-diOHprog in response to gonadotropin (Kanamori et al. 1988).

STEROIDAL MEDIATORS OF SPERMATOGENESIS AND SPERMIATION

Spermatogenesis and spermiation in teleosts have been considered to be under gonadotropin control (Clemens and Grant 1965, Billard et al. 1982). It generally is assumed, however, that gonadotropin does not act directly to induce spermatogenesis and spermiation in teleosts but works in concert with testicular somatic elements to stimulate the production of steroidal mediators. In male amago salmon, plasma levels of testosterone and 11-ketotestosterone are high during the later stages of spermatogenesis and rapidly decline after the onset of spermiation (Ueda et al. 1983a, Sakai, unpublished) (Figure 4). In contrast, the levels of 17α,20β-diOHprog are low during spermatogenesis and increase sharply at the time of spermiation (Ueda et al. 1983b) (Figure 4). These results suggest that, in amago salmon, androgens are involved in the later stages of spermatogenesis, whereas 17α,20β-diOHprog is involved in spermiation.

The involvement of 17α,20β-diOHprog in the process of spermiation was strongly supported by our studies demonstrating that a single injection of gonadotropin to non-spermiating amago salmon induced precocious spermiation 1 to 2 months prior to the normal spermiation period, concomitant with a marked increase in plasma levels of 17α,20β-diOHprog. Similarly, two successive injections of 17α,20β-diOHprog caused precocious spermiation, but the response to 17α,20β-diOHprog was of lesser magnitude than to gonadotropin. Neither

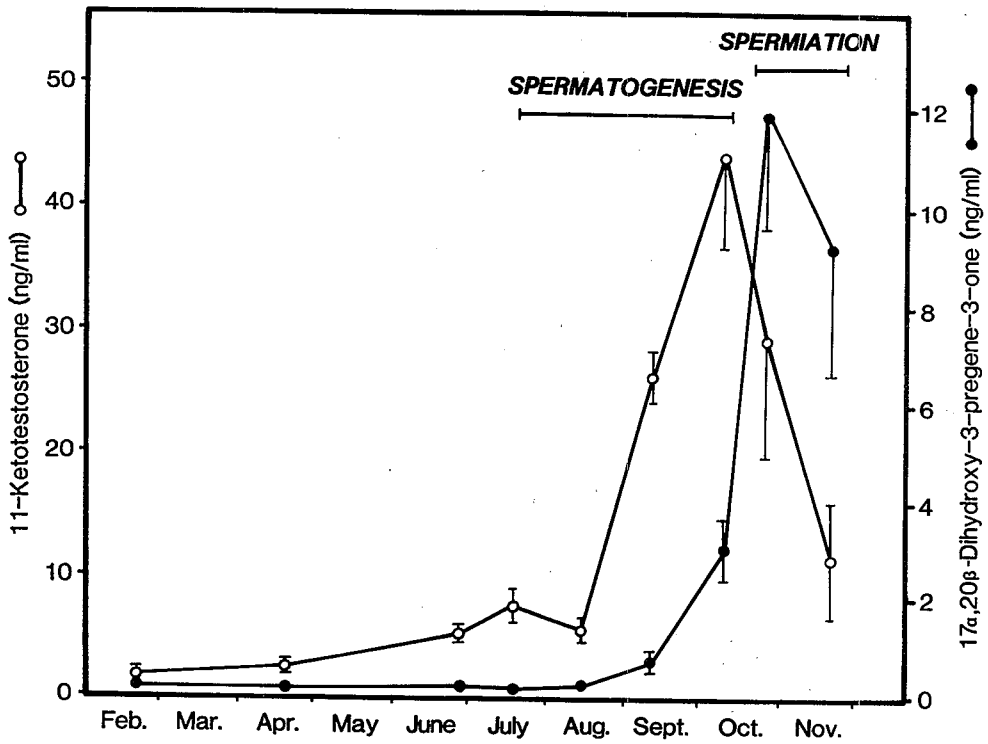

Figure 4. Changes in plasma 11-ketotestosterone and 17α,20β-dihydroxy-4-pregnen-3-one levels during sexual maturation of male amago salmon.

testosterone nor 11-ketotestosterone were effective (Ueda et al. 1985). Considered together, these results provide evidence to suggest that 17α,20β-diOHprog is a testicular steroidal mediator of gonadotropin-induced spermiation in salmonids. Similar suggestion has been made for goldfish (Kobayashi et al. 1986).

It seems most likely that the development of sperm motility in mammals is dependent on hormones, especially androgens (Hoskins et al. 1978). Nothing is known, however, about the role of hormones in the acquisition of sperm motility in teleosts. We have begun to explore this question by acquiring basic information about the effects of steroid hormones on the acquisition of sperm motility in salmonids. In masu salmon, sperm in the testis and sperm duct are not motile. If sperm duct sperm are diluted with fresh water, then for the first time, they gain motility. On the contrary, if testis sperm are diluted with fresh water, they will not become motile. Thus, two separate processes are involved in the induction of sperm motility in salmonids. One is the acquisition of motile ability when shifting from the testis to sperm duct, and the other is the initiation of motility when diluted with fresh water.

Early in the breeding season, however, sperm from the sperm duct do not gain motility even when they are diluted with fresh water. This

characteristic, namely the period during which sperm become motile, makes the masu salmon a good system for studying the endocrinological mechanisms involved in the acquisition of sperm motility. Effects of two successive daily intramuscular injections of three steroid hormones (17α,20β-diOHprog, testosterone, 11-ketotestosterone) on the acquisition of sperm motility were examined in masu salmon during the early breeding season. Injections of 17α,20β-diOHprog significantly raised the percentage of motile sperm and the duration of sperm motility. In contrast, the other two steroids showed no significant effect on both values. Similar stimulatory effects of 17α,20β-diOHprog on sperm motility were observed in the Japanese eel, Anguilla japonica. These results suggest that 17α,20β-diOHprog is involved in the acquisition of sperm motility in masu salmon and eel (Yamauchi et al. 1987). Further studies are currently under way to define the mechanisms by which 17α,20β-diOHprog injections stimulate sperm motility.

TESTICULAR STEROIDOGENESIS

In vitro incubation studies on 11-ketotestosterone production by testicular fragments at different stages of development have revealed that the capacity of the fragments to produce this steroid in response to gonadotropin is high during the later stages of spermatogenesis and rapidly declines after the onset of spermiation. In contrast, the capacity of testicular fragments to produce 17α,20β-diOHprog is low during spermatogenesis and increases sharply at the time of spermiation. Thus, a distinct shift in the steroidogenic pathway from 11-ketotestosterone to 17α,20β-diOHprog appears to occur in the testis of amago salmon around the onset of spermiation (Sakai et al., unpublished).

The preceding findings led to the conclusion that the testis is the principal source of 11-ketotestosterone and 17α,20β-diOHprog. Using three different testicular preparations (intact testicular fragments, sperm-free testicular fragments and sperm preparations), we examined the effects of gonadotropin, 17α-hydroxyprogesterone, and testosterone on 11-ketotestosterone and 17α,20β-diOHprog production. Incubation of intact or sperm-free testicular fragments with gonadotropin, 17α-hydroxyprogesterone or testosterone resulted in a highly significant increase in 11-ketotestosterone levels in the incubation medium. Sperm preparations did not produce 11-ketotestosterone at any incubation condition. Intact testicular fragments produced a large amount of 17α,20β-diOHprog in response to gonadotropin or 17α-hydroxyprogesterone.

Neither sperm-free testicular fragments nor sperm preparations alone were capable of producing 17α,20β-diOHprog in response to gonadotropin. 17α-Hydroxyprogesterone markedly stimulated 17α,20β-diOHprog production by sperm preparations, but not by sperm-free testicular preparations (Ueda et al. 1984). After incubation of intact testicular fragments with [^{14}C]17α-hydroxyprogesterone, 17α,20β-diOHprog and 11-ketotestosterone were identified as major metabolites (Figure 5). In contrast, only 17α,20β-diOHprog was identified when sperm preparations were incubated with [^{14}C]17α-hydroxyprogesterone. No further metabolite was obtained when sperm preparations were incubated with testosterone. These results indicate that different testicular elements are the sites of synthesis of 11-

ketotestosterone and 17α,20β-diOHprog. Furthermore, it is suggested the involvement of sperm in the production of 17α,20β-diOHprog in the testis of spermiating salmonids.

CONCLUSION

Inasmuch as 17α,20β-diOHprog was identified as the maturation-inducing hormone, we now have two known biologically important mediators of oocyte growth and maturation in salmonids, estradiol-17α and 17α,20β-diOHprog. It is now established that the granulosa cells are the site of production of these two mediators but production by the ovarian follicle depends on the provision of precursor steroids by the thecal cell layer (two-cell type model). A dramatic shift in the steroidogenic pathway from estradiol-17α to 17α,20β-diOHprog occurs only in ovarian follicle cells immediately prior to oocyte maturation. These results suggest that differential changes in the steroidogenic capacity of thecal granulosa cell layers are prerequisite for growing oocytes to enter the final stages of maturation. Resolution of the

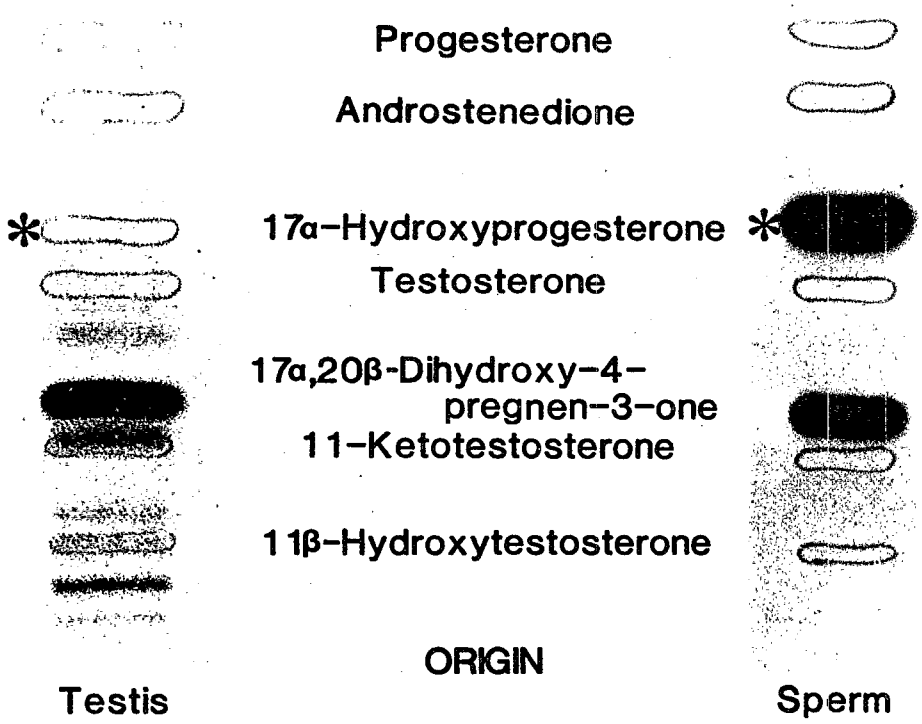

Figure 5. Metabolites of [^{14}C]17α-hydroxyprogesterone (*) by incubation of intact testicular fragments (Testis) and sperm preperations (Sperm) obtained from spermiating amago salmon. Authentic steroid standards are indicated.

molecular events regulating these changes may provide new insight into the hormonal events regulating oocyte growth and maturation.

17α,20β-DiOHprog also has been shown to be a major mediator of gonadotropin-induced spermiation in amago salmon. This steroid also was shown to be involved in the acquisition of sperm motility in masu salmon and eel. A shift in steroidogenic pattern from 11-ketotestosterone to 17α,20β-diOHprog seems to occur in the testis immediately prior to or during the spermiation period. Although the question of which cell type is the principal source of testicular production of 11-ketotestosterone and 17α,20β-diOHprog is still unresolved, it is likely that different testicular elements are the sites of synthesis of 11-ketotestosterone and 17α,20β-diOHprog. These findings may be one of the best documented examples of the relationship between testicular steroidogenesis and male germ cell maturation in nonmammalian vertebrates, and will certainly provide the basis for a study at the molecular levels of hormonal regulation of male germ cell development.

ACKNOWLEDGMENTS

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INDUCING EGG CELL FUSION WITH LASER

by

Ger Guo-chang[1], Zhang Wen-di[2], Zhau Pei[2], and Wang Qu[3]

INTRODUCTION

Cell fusion was first demonstrated with inactivated viruses by Okada (1960). A chemical method was established with polyethylene glycal (PEG) by Kao (1970), and an electrofusion method was developed by Zimmermann (1980). Such methods have several disadvantages, including a low viability rate for fused animal cells. The recent development of a laser microbeam technique, which has been especially successful in micro-surgery at the subcellar level for single living cells, offers a new cell fusion tool that could produce less damage than other methods. In our work, inducing egg cell fusion in the loach with the laser was successful. Fused eggs can cleave and develop. Some of them developed to gastrul stage; a few even to hatch stage and lived on.

A Nd: YAG laser of nano-second pulse and high peak power density is the basic source. A 0.53-um laser generated by second-harmonic crystal KDP and a 0.59-um laser generated by a dye laser, which is pumped by a 0.53-um laser, are employed as a working laser for microbeam irradiation of cells. An auxiliary He-Ne laser beam is for aiming. Both laser beams are collinearally focused through a microscope on the specimen. The radius of the spot affected by the laser can be adjusted by objective magnification of the microscope and the laser energy.

Breeding was induced with HCG out of the loach breeding season. After fertilization, the eggs were put into a medium containing 0.002 gram of pronase per milliter. Three minutes later, the membrane was peeled. The eggs were washed three times and placed in a 10% Holtfreter solution. The eggs then were loaded onto a special plastic plate that placed two adjacent eggs close together. The plate with the eggs was put on the stage of microscope. The He-Ne laser first was aimed at the contact region of two adjacent eggs and then the working laser beam was irradiated onto the eggs. The magnification of microscope objective and energy was just within the range in which the pore of membrane made by laser beam could reseal automatically. If the density of

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energy was too high, the membrane would be unable to reseal itself and the pore would be permanent so that the intercellular organelles would flow out of cells and affect the viability of the egg. The threshold should be measured by experiment. For fertilized loach egg, it was 10 uJ/um^2 by our practice. After the irradiation, the eggs are returned to the cultured medium and examined, and the development behavior is recorded.

RESULTS

After irradiation, the paired eggs at first appeared as a dumbbell. About 2 hours later (at 24°C), the fusing eggs were rounding into a spheroid, the lipid at the contact region between the two cells disappeared, and the whole volume was doubled compared with a normal egg. The two eggs were fused. The experiment has been repeated on 120 pairs of eggs; of these, 37 pairs were fused. Compared with the normal fertilization of eggs, the cleavage rate was 90% and the fusion rate was about 40%.

Most of the fused eggs cleaved. In two cases, the eggs did not cleave, and it was found that some of fused eggs cleaved rather slower than the normal egg cell.

The development patterns of embryo were divided into two types, depending on the relative position of the two blastodiscs in the fused eggs.

1. The two blastodiscs were so far apart that they could not join together. Most of the fused eggs belonged to this type. For most of them, development was arrested prior to the blastula stage. Some of the further developing embryos apparently maintained separative cleavage; a few of them developed to mophopoiesis. For the others, one of the two blastodiscs ceased cleavage at certain stage of development; another continued to develop further.

2. The two blastodiscs were on the same side and close to each other. They bean to cleave separately. But as the number of blastomeres gradually increased, they joined together and continued to develop to single blastula, gastrula, embryonic coelm, even newly hatched larvae.

The number of embryos developing to various stage are listed in Table 1.

TABLE 1. NUMBER OF EMBRYOS DEVELOPING TO VARIOUS STAGES

Development type	Cleavage	Blastula		Gastrula	
		Single	Double	Single	Double
Larva					
1	27	9		5	1
			4	1	3 1
2	8	5		3	2

In on-going work in 1988, three pairs of fused eggs developed--one into a normal larvae, with one head and one tail, normal in external structure. It developed faster than the unfused control egg, however, and lived for 10 days. The other two also hatched--one with two heads and one tail, the other with the tail fused together, but with two separate bodies. They died in a few days.

DISCUSSION

The results showed that the larvae, fused from two eggs by laser developed well and that there was no margin in the middle of the body. The unfused fish could join two bodies into one, but without laser inducing, there was a very clear margin within the body that separated the fish into two. It also was found that the size of the fused egg was bigger than a normal one. The diameter of a normal egg was 0.925 mm and the volume of it was 0.31 mm^3. The fused one was 1.175 mm in diameter and 0.63 mm^3 in volume. It was about twice the size of a normal egg.

The laser inducing fusing could occur only when two closed egg cells were arranged closely, and the two blastodiscs were on the same side and close. If the two blastodiscs were on contrary sides, or if one was up and the other was down, the fused egg cell could only develop into an abnormal fish with two heads or other deformities.

The wavelength of the working laser used in our experiment was 530 nm and 590 nm. In another experiment, a laser beam with a wavelength of 266 nm was used to treat the fertilized eggs of the loach. Results showed that it was harmful to the cell and caused many embryo variations. So in this experiment, the wavelength of the working laser used was 530 nm, 590 nm to avoid any variations, and it was found it was not harmful for the cell--the embryo developed normally.

The technique did not work on the walking catfish. The inner pressure of the catfish egg was higher than loach. After laser irradiation, the eggs were broken. The method and dosage of laser for fish egg cell fusion needs more research.

SUMMARY

The results showed that the laser-induced cell fusion is available in fertilized fish eggs, and a high survival rate was achieved. It was considered that the method is new and valuable for egg cell fusion. The work is still going on with more analysis and research on embryo development and egg fusion needed.

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TESTOSTERONE AND 11-OXOTESTOSTERONE CHANGES DURING AN ANNUAL CYCLE AND INDUCED SPAWNING IN BLUNT SNOUT BREAM (MEGALOBRAMA AMBLYCEPHALA)

Zhao Wei-xin[1]

INTRODUCTION

Higher vertebrate androgen comes from two parts, adrenal cortex and gonads (testis and ovary), but the testis is the major place where androgen is synthesized and excreted. Among the excreted androgens, the biological activity of testosterone is the highest. The testis of fish can synthesize not only testosterone and androstenedione but also another androgen, 11-oxotestosterone (11-OT or 11-ketotestosterone, 11-KT), which has very high activity. The 11-OT level in blood of Atlantic salmon (Salmo salar) is much higher than the testosterone level (Hunt and Wright 1981). In Atlantic salmon (Hunt and Wright 1981), rainbow trout (Salmo gairdneri) (Schulz 1984, Scott et al. 1980, Simpson and Wright 1977) and goldfish (Carassius auratus) (Kobayashi et al. 1986), testosterone levels in blood of female fish are higher than those of male fish of the same species. This also is observed in female amphibians and reptiles. The 11-OT level in blood of female fish is very low, however (Simpson and Wright 1977, Wright 1976). In this experiment, we investigate the changes of androgen excreution and attempt to discuss the physiological function of androgen during the annual reproductive cycle based on the studies of oestradiol by the author in 1986.

MATERIALS AND METHODS

Sampled fish were the first sexual maturation of blunt snout bream (Megalobrama amblycephala) collected from a pond at the Wujiang County Fisheries Institute in Jiangsu province. Artificially induced spawning had not been used and the fish had not spawned spontaneously. Study fish (7-13 male and 9-14 female fish weighing 250 to 700g) were taken randomly. Blood samples were removed from the caudal vein of each fish at the end of each month from January to December 1984. The serum was collected and stored at -40°C until assay. At same time, body and gonad weights of sampled fish were recorded, and the gonadsomatic index (GSI-gonad wt. x 100/body wt.) was determined.

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Spawning was induced with human chorionic gonadotrophin (HCG) (1500 I. U./kg body wt. for female, a half dose for male) or HCG plus dried pituitary gland of carp (500 I. U. HCG + 1mg CPG/kg body wt. for female, a half dose for male) in May 1985. Blood samples were taken before and after injection, and the serum was collected and stored at -40°C until assay.

Radioimmunoassay (RIA) was used to measure serum T and 11-OT levels according to the method of Zhao Wei-xin (1987). ^{3}H-testosterone (^{3}H-T) and ^{3}H-11-oxotestosterone (^{3}H-11-OT) are the products of British Amersham Company. T standard and T antiserum were obtained from Shanghai Institute of Endocrinology, 11-OT standard came from Sigma Company, 11-OT antiserum was provided by Dr. R. S. Wright of the Marine Laboratory, Aberdeen UK. The ranges of T and 11-OT standards are 0 to 400 pg and 0 to 600 pg, respectively. A method of comparison between heating and extraction was made during T and 11-OT measurements; no significant difference was found ($P > 0.05$).

RESULTS

The peak level of T (2.45 ± 0.35 ng/ml, X ± SE) was shown in March, a month before GSI peak (see Figure 1). The T level then declined to 1 to 2.5 ng/ml from April to July with a minimum closing to base line level in August. The second peak (1.17 ± 0.06 ng/ml) was found in November. At that time, GSI rose slightly. The T level declined again in December. The annual change of serum T levels was not correlated with the annual change of GSI, r-0.2892 ($P > 0.05$).

The peak level of 11-OT (32.84 ± 5.30 ng/ml) was shown in April, in accordance with the peak level of GSI. The 11-OT level was 14.30 ± 1.15 ng/ml in May, about a half-peak level. 11-OT level decreased continuously to a minimum (0.31 ± 0.05 ng/ml) in September. The annual change of 11-OT levels was highly correlated with the annual change of GSI, $r=0.8355$ ($P < 0.01$).

Five mature male and female fish were injected with HCG and then put into a concrete-lined pool (water temperature 25°C). After 12 hours, two females had ovulated, one had spawned, and two had neither ovulated nor spawned. At this time, serum T level of males rose from basic line level before injection to 1.53 ± 0.62 ng/ml (Figure 2). A high-significance difference ($P < 0.01$) was found during induced spawning. The serum 11-OT level of males was 4.49 ± 0.35 ng/ml (n-4) 12 hours after injection (Figure 3). No significant difference ($P > 0.05$) was found during induced spawning although the T level showed a tendency to decrease.

For the year (Figure 4), the T level was minimum (0.49 ± 0.08 ng/ml) in January and then rose gradually. After March, the T level increased rapidly. The peak level (5.03 ± 0.45 ng/ml) was shown in April. The T level declined slightly in May and decreased sharply in June. A small peak was shown in July (2.53 ± 0.26 ng/ml). There was no obvious change of T level from August to December (0.7 to 1.2 ng/ml). The annual changes of T level was correlated with the annual change of GSI, $r=0.5151$ ($P < 0.05$).

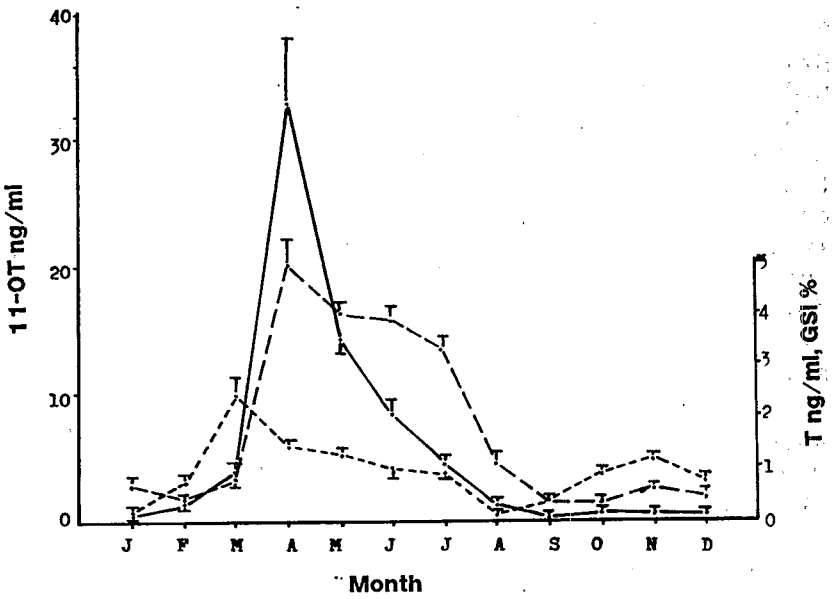

Figure 1. Annual changes in serum T, 11-OT and GSI in male blunt snout bream (----T, ———11-OT, — — —GSI).

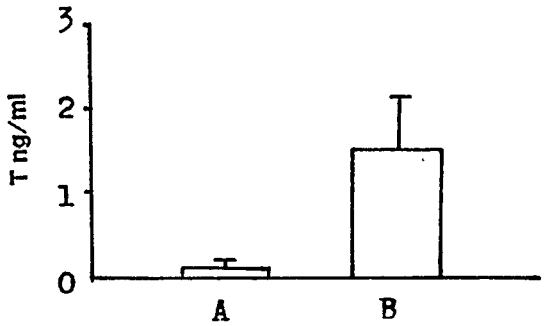

Figure 2. Changes in serum T level during induced spawning in male blunt snout bream (A--before injection, B--24 hours after injection).

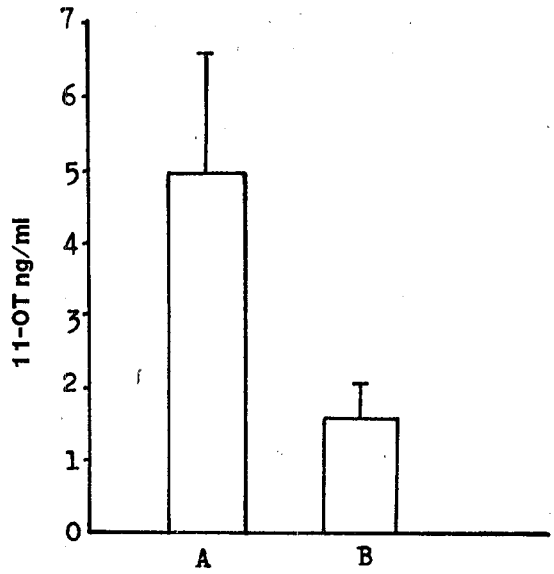

Figure 3. Changes in serum 11-OT level during induced spawning in male blunt snout bream (A--before injection, B--12 hours after injection).

Changes for the first group (in Qinpu Freshwater Fish Farm in May 1985 are shown in Figures 5 and 6. The mature male and female fish were injected with HCG + CPG and then put into a concrete-lined pool (water temperature 22°C). After 14 hours, males had produced milt; females had not spawned or ovulated. Measurements of serum T and 11-OT levels during induced spawning indicated that the T level was 1.95 ± 0.53 ng/ml (n=10) in the female before injection and that the T level increased rapidly to 17.96 ± 4.32 ng/ml (n=10) 14 hours after injection. A highly significant difference ($P < 0.01$) was found during induced spawning. Serum 11-OT level in females was very low (0.98 ± 0.08 ng/ml) (n=7) before injection and increased to 1.53 ± 0.18 ng/ml (n=7) 14 hours after injection. A significant difference ($P < 0.05$) was found during induced spawning.

From the second group (in Wujiang County Fisheries Institute, June 1985), five mature male and female fish were injected with HCG before being placed in a concrete-lined pool (water temperature 25°C). After 12 hours, three females had spawned or ovulated. At this time, the average T level of three spawners was 8 ng/ml and the T levels of two non-spawners were all over 10 ng/ml.

DISCUSSION

It is generally thought that T has an effect on spermatogenesis. The metabolic substances of T, released out of the body with urine, can attract the opposite sex and affect sexual behavior. In addition, T is a precursor of estrogen biosynthesis. T may promote gonadotrophin (GTH) to stimulate sperm release and ovulation. T has synergism with GTH and strengthens GTH, inducing

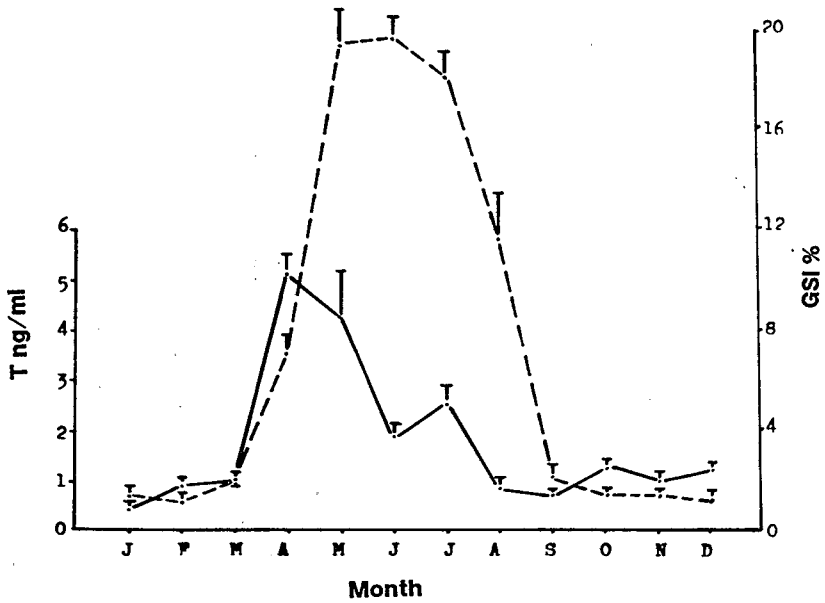

Figure 4. Annual changes in serum T level and GSI in female blunt snout bream (———T,— — —GSI).

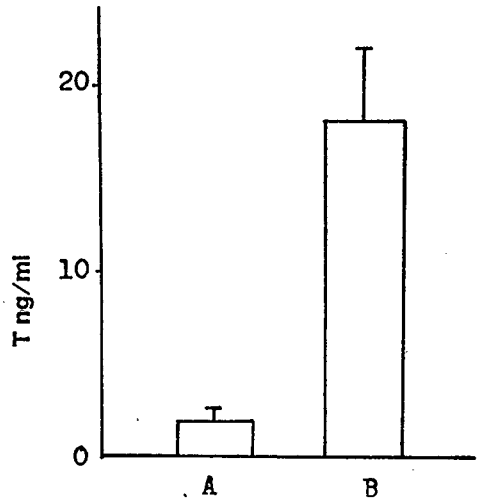

Figure 5. Changes in serum T level during induced spawning in female blunt snout bream (A--before injection, B--24 hours after injection).

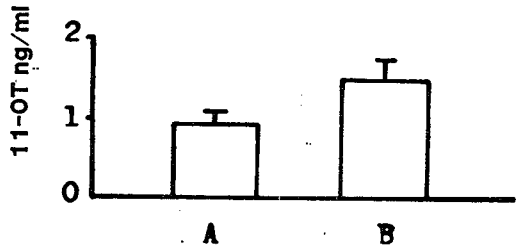

Figure 6. Changes in serum 11-OT level during induced spawning in female blunt snout bream (A--before injection, B--14 hours after injection).

17α-hydroxy-20ß-dihydroprogesterone (17α-20ßP) production. 17α-20ßP is considered to be an effective maturation-inducing hormone released by testis and ovary.

Our results showed that the GSI of male fish reached its maximum in April (Figure 3). The serum T level increased obviously after January; the peak level of T was about a month earlier than that of GSI. This indicated that T stimulated testis development and spermatogenesis. After September, T level increased again and formed a small peak in Autumn. It may be associated with the testis development of the next sexual cycle and stimulate the spermatogonia proliferation and spermatocyte formation. In point of sexual cycle of female fish (Figure 6), the peak level of T appeared in April, also about a month earlier than maximum GSI of female fish. A higher level of T was maintained in May (reproductive season).

During a female sexual cycle, the T level alternated between 1 and 5 ng/ml and was above 2 ng/ml for 4 months in a year. But during a male sexual cycle, the T level was lower, only between 0.2 and 2.5 ng/ml. This also was found in salmonid--the T level was higher in female than that in male. This may closely associate T as a precursor of estrogen synthesis. In blunt snout bream, the annual change of E_2 was similar to that of T (Wright 1976). The peak level of E_2 appeared in April, followed by a lower peak in June, and a still smaller peak maintained from October to December. These peaks indicated that the changes of E_2 synthesis and release are closely related to the changes of T level in the annual cycle.

The T level increased in both sexes after hormone injections. In the female, the T level greatly increased after injection, and decreased during

ovulating or spawning. This change also was found in salmonid (Zhab et al. 1987). At this time, T is not viewed as a precursor of E_2 synthesis. It seems to be a synergist that acts with greatly released GTH to induce steroid production and lead to germinal vesical movement, polarization, and disappearance. The metabolite of T, a kind of 5ß-reduced androgen, was released as a sex pheromone to attract male fish. In the male, the T level also increased. The T level decreased after injection in male silver carp (Hypophthalmichthys molitrix) found by author (unpublished). The changes of T level during induced spawning requires further study.

It generally is thought that 11-OT markedly stimulates the development of secondary sex characteristics in males. In females, the 11-OT level was very low and usually not able to be determined. Therefore, 11-OT has been used to identify the sexes before the appearance of secondary sex characteristics (Wright 1976). In our experiment, the annual changes of male GSI, T and 11-OT (Figure 6), showed that the releasing content of 11-OT increased as GSI increased gradually. The serum 11-OT level rapidly increased 1 or 2 months before reproductive season (May) and the secondary sex characteristics of male were becoming apparent. The peak level of 11-OT occurred in April, simultaneous with the peak of GSI. The 11-OT peak level was about 13 times greater than the peak T level. T synthesized and released in the Spring may be used to stimulate spermatogenesis and to serve as a precursor converting into 11-OT. In the late period of spermatogenesis (April), however, T mainly converts into 11-OT.

There was a tendency for 11-OT to decrease in the male with hormone injection, but no significant difference was found in comparison with pre-injected. This was similar to the author's results (unpublished), which were obtained by culturing mature testis in vitro. This indicates that 11-OT has no direct effect on sperm release (Fostier et al. 1984). The 11-OT level increased in females after hormone injection. This may be caused by the increase of T level and the increase of converting into 11-OT at this time. 11-OT may have no direct effect on oocyte final maturation.

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NUTRIENT REQUIREMENTS OF JUVENILE ALLOGYNOGENETIC CRUCIAN CARP, CARASSIUS AURATUS GIBELIO

by

He Xiqin[1], Jia Lizhu, Li Zhongjie, and Yang Yunxia

INTRODUCTION

A nutritionally well-balanced and low-cost formulated diet is essential to fish farming, especially to the intensive culture of high quality food fish. A reasonable feed formulation and high efficiency diet can only be based on an understanding of the nutrient requirements of cultivated fish. On the other hand, because the feed represents up to 50% of aquaculture expense, one cannot afford the economic loss caused by improper feed and feeding regimens. Therefore, the study of nutrient requirements of fish is important both in basic fish nutrition and in practical fish production.

Since DeLong et al. studied the optimal protein level in food for chinock salmon, Oncorhynchus tshawytscha, in 1958, the literature on nutrient requirements in fish has grown rapidly (Millikin 1982a, NRC 1983). So far, the results show that the protein requirements range from 23 to 55% in different fishes. Because of different diet compositions and experimental conditions, there are marked differences in protein requirements even within the same fish species. For example, requirements for the grass carp, Ctenopharyngodon idella, have been reported as 27.66% (Lin Ding et al. 1980), 36.7% (Mao Yongqing et al. 1985a), and 41 to 43% (Dabrowski 1977). Consequently, the determination of nutrient requirements of fish is not a matter of simple measurement but a complicated problem involving many factors.

Crucian carp is a commercially important food fish in China. Although crucian carp successfully maintain large populations in ponds and lakes, they take 3 year to grow to market size (250 g). Allogynogenetic crucian carp is an improved breed obtained by using heterologous sperm to initiate the nucleus development of female crucian carp. The new breed exhibited good growth performance in the ponds, cages, and lakes. Little is known, however, about the nutrient requirements of this species. Our experiment was aimed at investigating the optimum levels of dietary protein, fat, carbohydrate, and cellulose that would promote the maximum growth of juvenile allogynogenetic crucian carp.

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MATERIALS AND METHODS

An orthogonal design method was employed in two experiments for a period of 30 days each. Nine experimental diets with different levels of nutrients were prepared according to the orthogonal table $L_8(3^4)$. A nonprotein diet was arranged so that NPU could be calculated by the method of Miller and Bender (1955). The factors and levels being examined and the composition of experimental diets are shown in Tables 1 and 2, respectively. Experimental diets were formulated by using purified food. Casein, corn oil and fish liver oil, potato starch and dextrin, and cellulose were used as protein, fat, carbohydrate, and cellulose sources, respectively. Mineral and vitamin premix were prepared according to NRC methods (1977, 1983).

The test fish were provided by an aquaculture experiment station and reared in the fish pond before use. Before the start of the experiment, the fish were transferred to indoor, 57 x 33 x 32 cm plastic aquaria of 40-liter capacity. Fifteen fish were stocked in each aquarium and fed with commercial feed containing 30% protein. Tap water was filtered with activated charcoal, heated, aerated, and run into each aquarium at the rate of 0.6 l/min. Test diets were fed according to the quantity designed by the orthogonal table in experiment I and fed at 3 to 4% of fish wet weight daily in Experiment II. The fish were fed three times a day at 0800, 1200 and 1600 hours. All fish were weighed every 10 days in order to regulate the feeding quantity or rate. In Experiment I, the average body length was 4.25 ± 0.17 cm, with an average body weight of 2.53 ± 0.31 g. In Experiment II, the average body length was 5.0 ± 0.4 cm, and the body weight was 3.58 ± 0.20 g. The water had a temperature of 23.1 ± 0.8°C and 28.8 ± 0.6°C, a dissolved oxygen content of 5.43 ± 0.83 mg/l and 5.29 ± 0.71 mg/l, and a pH of 6.9 to 7.1 in Experiments I and II, respectively.

TABLE 1. NUTRIENTS AND THEIR LEVELS IN EXPERIMENT I AND II

(g/100g body weight/day)

	Experiment I				Experiment II		
Level	Protein	Fat	Carbohydrate	Cellulose	Protein	Fat	Carbohydrate
1	0.8	0.1	0.7	0.3	1.1	0.07	0.93
2	1.2	0.2	0.9	0.4	1.3	0.17	1.11
3	1.6	0.3	1.1	0.5	1.5	0.26	1.29

TABLE 2. COMPOSITION OF THE EXPERIMENTAL DIETS (g/100 g body weight/day[a])

Diet number	Experiment I				Experiment II		
	Protein	Fat	Carbohy-drate	Cellu-lose	Protein	Fat	Carbohydrate
1	0.8	0.3	0.7	0.4	1.1	0.26	0.93
2	1.2	0.1	0.9	0.3	1.3	0.07	0.93
3	1.6	0.2	1.1	0.5	1.5	0.17	0.93
4	0.8	0.2	0.7	0.3	1.1	0.17	1.11
5	1.2	0.3	0.9	0.5	1.3	0.26	1.11
6	1.6	0.1	1.1	0.4	1.5	0.07	1.11
7	0.8	0.1	0.7	0.5	1.1	0.07	1.29
8	1.2	0.2	0.9	0.4	1.3	0.17	1.29
9	1.6	0.3	1.1	0.3	1.5	0.26	1.29

[a]6% mineral premix, 3% vitamin premix and 1% binder (taro powder) were added to each diet.

Twenty fish at the beginning and at the end of each treatment were taken for carcass analysis. Crude protein was measured by Kjedahl's method, crude fat by Soxhlet ether extract, ash by burning at 600°C for 12 hours, crude fiber by acid-base washing method, dry weight by drying at 105°C for 24 hours, and carbohydrate by the indirect method of difference. The obtained data were compared directly with the range values of orthogonal design.

RESULTS AND DISCUSSION

NUTRIENT REQUIREMENTS

The results of Experiment I are shown in Table 3 and Figure 1. Among the nutrients, the change of dietary protein level played the most important role in the growth of juvenile allogynogenetic crucian carp. The range value was 0.45%. Carbohydrates and cellulose were less important for growth and the range values were 0.21 and 0.15% respectively. The dietary fat level seemed

TABLE 3. EFFECTS OF NUTRIENTS AND THEIR LEVELS ON SGR, FCR, PDR, PER AND NPU OF JUVENILE ALLOGYNOGENETIC CRUCIAN CARP FOR EXPERIMENT I

	Specific growth rate (SGR)				Feed conversion ratio (FCR)				Protein deposition rate (PDR)				Protein efficiency ratio (PER)				Net Protein[a] utilization (NPU)			
Level	P	F	S	C	P	F	S	C	P	F	S	C	P	F	S	C	P	F	S	C
1	1.06	1.34	1.24	1.30	3.28	2.28	3.19	2.91	25.0	32.2	24.0	27.9	0.56	0.55	0.46	0.58	47	49	40	45
2	1.41	1.32	1.28	1.41	2.65	3.08	3.05	2.82	33.6	27.8	24.4	30.3	0.71	0.61	0.58	0.64	50	45	42	46
3	1.51	1.32	1.45	1.26	3.03	2.98	2.74	3.11	25.0	23.6	35.1	25.4	0.50	0.61	0.72	0.55	37	40	45	43
Range	0.45	0.02	0.21	0.15	0.63	0.80	0.45	0.29	8.6	8.6	11.1	4.9	0.21	0.06	0.26	0.09	13	9	12	3
Better Level	P3	F1	S3	C2	P2	F1	S3	C2	P2	F1	S3	C2	P2	F2,3	S3	C2	P2	F1	S3	C2
Factor sequence	P S C F				F P S C				S P F C				S P C F				P S F C			

[a] SGR = lnWt - lnWO/t(days); FCR = dry feed intake/weight gain; PDR = 100 x body protein gain/protein intake; PER = wet weight gain/protein intake; NPU = carcass-N increased - endogenous loss of N/protein-N consumed.

[b] P,F,S,C represent protein, fat, carbohydrate and cellulose, respectively.

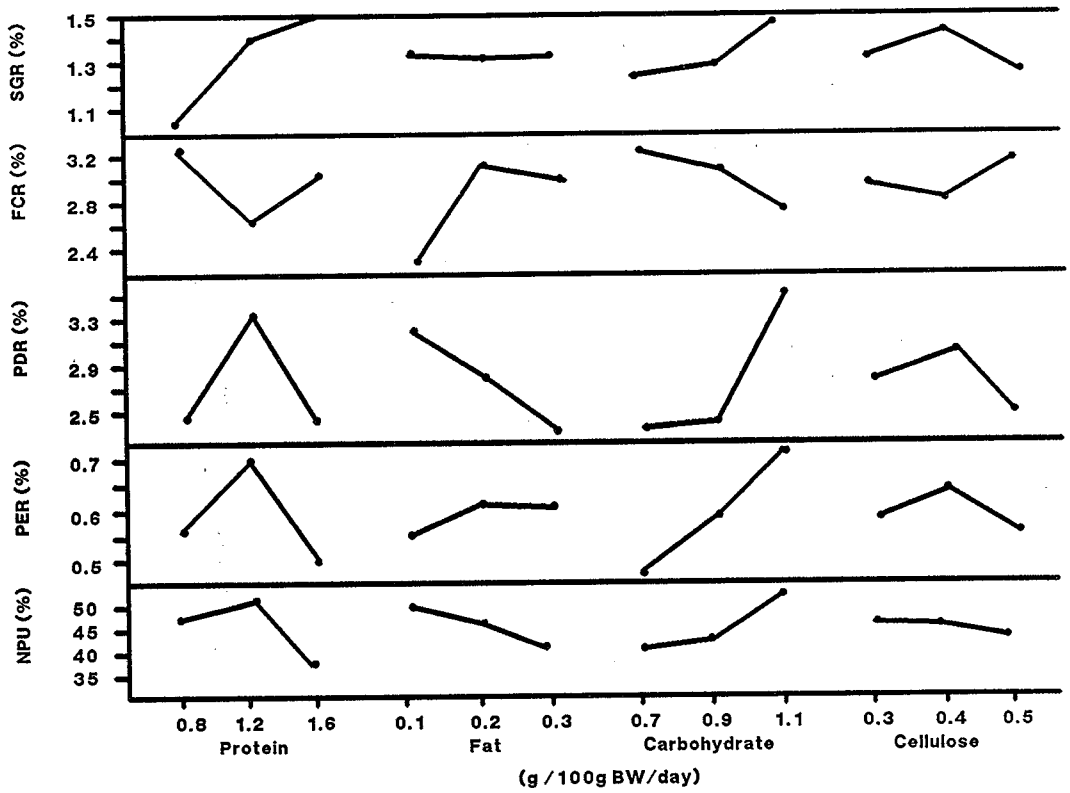

Figure 1. Relationships between dietary nutrient levels and SGR, FCR, PDR, PER, and NPU of juvenile allogynogenetic crucian carp for experiment I.

to have no definite effects on the growth. The range value was only 0.02%. As shown in Figure 1, daily SGR increased proportionally with increasing dietary protein level. The rate of growth increased rapidly while protein level increased from 0.8 g to 1.2 g and the increase of growth rate slowed with further increase in protein intake. The lowest FCR and the highest PDR, PER and NPU fell on the second level of dietary protein, i.e., 1.2 g. These results indicated that the optimum protein level lay between 1.2 and 1.6 g. Although the fat level seemed to have no definite effect on SGR, the FCR increased and the PDR and NPU decreased with the increasing dietary fat levels. The optimum dietary fat level was 0.1 g. The dietary carbohydrate level played a less important role on the growth of fish. An increase in SGR, PDR, PER, and NPU and a decrease in FCR were observed as the dietary carbohydrate level increased. The optimum dietary carbohydrate level was 1.1 g. Because the optimum fat and carbohydrate level lay on the margin of the test range, it is necessary to extend the test level range. The optimum dietary cellulose level was noted at the middle level, i.e., 0.4 g. The growth rate and the utilization of feed and protein decreased on either side of cellulose level. So there is no need to examine the cellulose level further. The results from Experiment I indicated that the nutrient requirements were: protein, 1.2 to 1.6 g; fat, 0.1 g; carbohydrate, 1.1 g; and cellulose, 0.4 g/100g body weight/day.

The results of Experiment II are shown in Table 4 and Figure 2. The value for dietary protein level was highest in all the parameters examined. Dietary protein also played the main role in the diet of fish; carbohydrate and fat ranged next in importance. As shown in Figure 2, the highest SGR, PDR, PER, and NPU and the lowest FCR occurred at the middle level of dietary protein, i.e., 1.3 g. No differences in growth rate and little difference, if any, in FCR value between fat levels of 0.17 g and 0.26 g were observed. However, the PDR, PER, and NPU were better in 0.17 g than in 0.26 g. So it could be considered that the optimum fat level was 0.17 g. There was not much increase in SGR (only 0.02%), as the carbohydrate increased from 1.11 to 1.29 g. On the other hand, FCR increased and PER and NPU decreased as the carbohydrate level increased. The optimum dietary carbohydrate level was 1.11 g.

On the basis of this study, it can be concluded that, under the experimental conditions, the nutrient requirements of juvenile allogynogenetic crucian carp were: protein, 1.3 g; fat, 0.17 g; carbohydrate, 1.1 g; and cellulose, 0.4 g. Transferring to percent content of nutrients, the diet should contain 39.3% protein, 5.1% fat, 36% carbohydrate (including 2.5% of potato starch in the vitamin premix) and 12.1% cellulose with an additional 6% mineral premix, 0.5% vitamin, and 1% binder. The fish obtained maximum growth when a diet formulated according to this composition was fed to the fish at the daily rate of 3.3% body weight.

EFFECTS ON FISH BODY COMPOSITION

The carcass composition between the fish at the end and at the start of the experiment are shown in Figure 3. Dry matter and ash content in the fish at the end were 2.2% and 11.4% lower; protein and fat content in the fish at the end were 1.4% and 7.8% higher than the fish at the start. In fish fed with the nonprotein diet, protein and ash content decreased dramatically, being 17.4% and 17% lower, and fat and dry matter content increased markedly, being 34.9% and 12% higher than that of the initial specimens.

The effects of nutrients and their level on the body composition are shown in Table 5. There was no significant difference in body composition due to the different diets. The range values of protein for gross body composition were 0.6 to 0.8%, thus there was no definite correlation between the protein content of the fish and the diets. This result agrees with the results for channel catfish, Ictalurus punctatus, (Page and Andrew 1973); striped bass, Morone saxatilis, (Millikin 1982b); and red drum, Scaiaenops ocellatus, (Daniels and Robison 1986) but differs from the results for plaice, Pleuronectes platessa, (Cowey et al. 1972); and rainbow trout, Salmo gairdneri, (Satia 1974, Austreng and Refatie 1979).

The lack of correlation between body protein and diet protein is probably either due to the fact that duration of the experiment was not long enough (Millikin 1982b) or that the selected test range is too close to the optimum level (Papaparaskeva-Papoutsoglou and Alexis 1986). The dietary fat level, however, seemed to have some effect on the fish body composition. Dietary fat was negatively correlated with body protein content and positively correlated with body fat content (Figure 4). The regression of body protein

TABLE 4. EFFECTS OF NUTRIENTS AND THEIR LEVELS ON SGR, FCR, PDR, PER AND NPU OF JUVENILE ALLOGYNOGENETIC CRUCIAN CARP FOR EXPERIMENT II

	Specific growth rate (SGR)			Feed conversion ratio (FCR)			Protein disposition rate (PDR)			Protein efficiency ratio (PER)			Net protein utilization (NPU)[a]		
Level	P	F	S	P	F	S	P	F	S	P	F	S	P	F	S[b]
1	1.02	1.17	1.15	4.23	3.77	3.68	43.7	44.1	42.1	0.72	0.71	0.74	81	75	77
2	1.37	1.25	1.25	3.14	3.52	3.45	54.3	51.5	48.5	0.89	0.79	0.82	87	85	82
3	1.28	1.25	1.27	3.34	3.42	3.58	43.1	45.5	50.5	0.71	0.75	0.77	72	72	81
Range	0.35	0.08	0.12	1.09	0.35	0.23	11.2	7.4	8.4	0.18	0.08	0.08	15	13	5
Better level	P2	F2,3	S3	P2	F3	S2	P2	F2	S3	P2	F2	S2	P2	F2	S2
Factor sequence	P S F			P F S			P S F			P S F			P F S		

a and b are the same as Table 2.

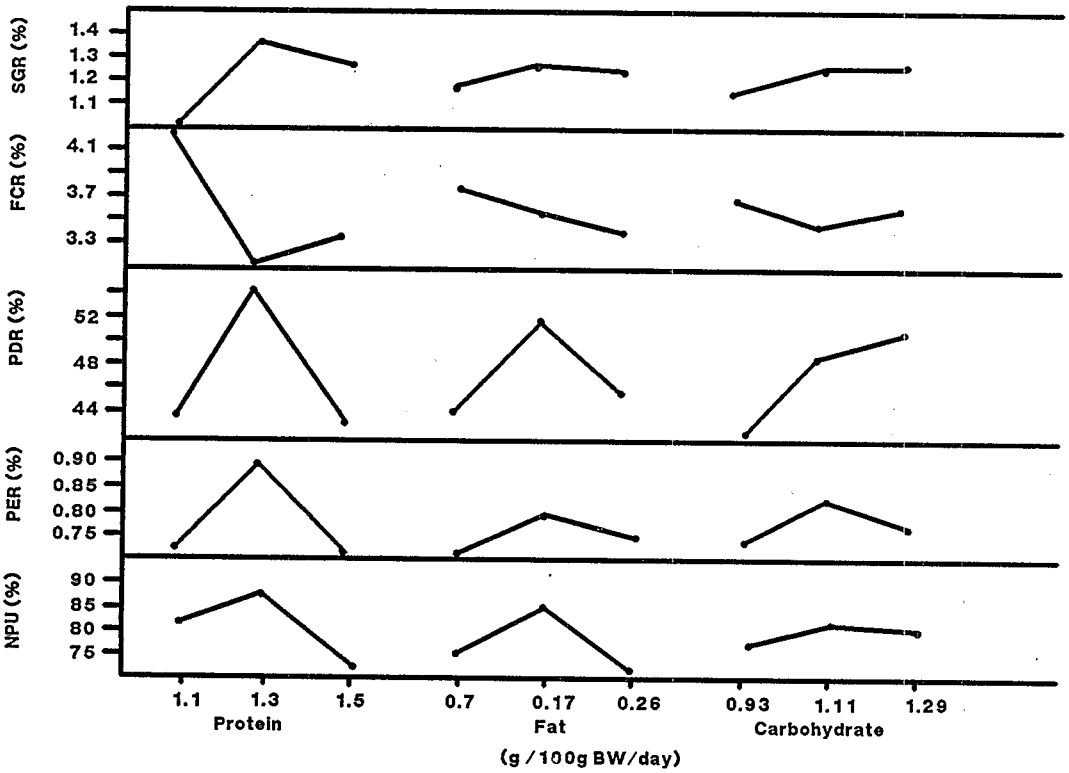

Figure 2. Relationships between dietary nutrient levels and SGR, FCR, PDR, PER, and NPU of juvenile allogynogenetic crucian carp for experiment II.

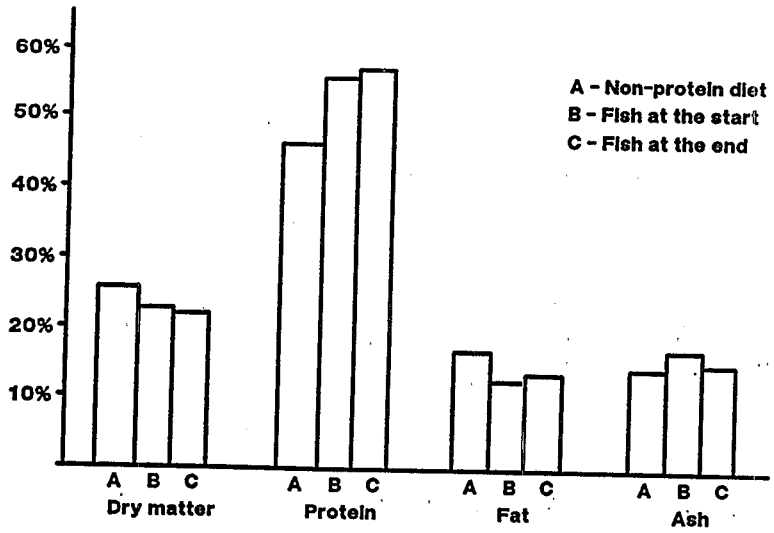

Figure 3. Comparison of carcass composition at the start and at the end of the study.

TABLE 5. EFFECTS OF NUTRIENTS AND THEIR LEVELS ON BODY COMPOSITION OF JUVENILE ALLOGYNOGENETIC CRUCIAN CARP

	Dry Matter (%)				Protein (5%)				Fat (%)				Ash (%)			
Level	P	F	S	C	P	F	S	C	P	F	S	C	P	F	S	C[a]
1	22.4	22.0	22.1	21.8	57.3	58.9	58.0	58.0	13.8	13.2	13.7	13.4	16.1	16.0	16.2	15.6
2	22.0	22.3	22.2	22.1	56.6	57.1	56.4	56.9	14.2	14.1	13.7	13.9	15.4	15.8	15.9	15.6
3	21.8	21.9	21.9	22.4	57.4	55.2	56.8	56.3	13.6	14.4	14.3	14.3	15.4	15.1	15.7	0.5
Range	0.6	0.4	0.3	0.3	0.8	3.7	1.6	1.7	0.6	1.2	0.6	0.9	0.7	0.9	1.4	0.1
Better Level	P1	F2	S2	C3	P3	F1	S1	C1	P2	F3	S3	C3	P1	F1	S1	C3
Factor sequence	P F S C				F C S P				F C P S				S F P C			

[a] P,F,S,C represent protein, fat, carbohydrate and cellulose, respectively.

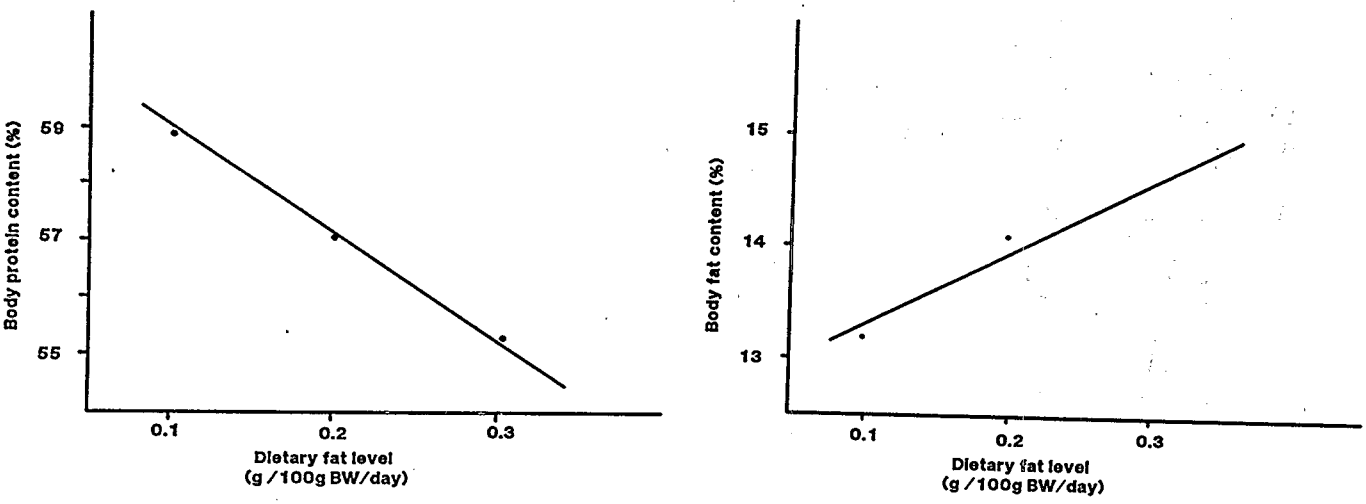

Figure 4. Relatinships between dietary fat level and body protein content (left) and body fat content (right).

content on dietary fat level and body fat content on dietary fat level were calculated and the equations obtained are $Y = -18.5x + 60.77$ ($r = 0.999$), and $Y = 6.00x + 12.7$ ($r = 0.961$). This result agrees well with previous studies (Satia 1974, Daniels and Robison 1986, Watanabe 1982). The body ash content decreased with the increase of dietary carbohydrate level (Figure 5). The regression equation is $Y = -3.5x\ 18.78$ ($r = 0.949$).

DETERMINATION OF NUTRIENT REQUIREMENTS OF FISH

Traditionally, the response of the fish to the graded level of single nutrient is evaluated for the determination of nutrient requirements of fish. For this method, all factors must be the same between treatments except the one to be evaluated. Numerous studies of nutrient requirements of fish have been performed by this method. However, the method is time and labor intensive and costly.

The orthogonal design method examining multiple factors has been used in this study. This method arranged the test reasonably by using a prepared, regularized table to evaluate the nutrient requirements under the interrelated effects of multiple factors. In Experiment I of this study, the fish were fed according to the quantity designed by the orthogonal table. Even though the exact amount of nutrients was offered to the fish, the quantity of feed intake by the fish is different between different test diets. A sense of hunger may emerge by those fish with low feed intake. In an attempt to overcome this problem, more cellulose was added to fill up the diets and then all fish were fed with the same feeding rate in Experiment II. Obviously, more investigation is needed concerning the application of the orthogonal design method.

To check whether the two differently designed method produced similar test results, the determination of dietary protein requirement by using the graded design method has been conducted in parallel under the same test condition as the orthogonal design method. Results are shown in Figure 6. SGR increased proportionally with increasing dietary protein level up to 40%. Beyond this level there was a slight retardation of the growth. This growth pattern in response to the dietary protein level agrees with results for eel, Anguilla japonica, (Nose and Arai 1972); plaice (Cowey et al. 1972); grass carp (Dabrowski 1977); tilapia, Tilapia zillii, (Mazid et al. 1979); and black carp, Mylopharyngodon piceus, (Yang Guohua et al. 1985), but different from the results for chinock salmon, (DeLong et al. 1958); carp, Cyprinus carpio (Ogino and Saito 1970) gilthead bream, Chrysophrys aurata, (Sabaut and Laquet 1973); and rainbow trout (Satia 1974). The relationship between dietary protein content and the growth rate of juvenile allogynogenetic crucian carp can be expressed by a parabolic curve and the polynomial regression formula was $Y = 0.0975 + 0.0223x - 0.000173x$ ($r = 0.874$). Obviously, the results obtained by both experimental design methods were almost the same. Used properly, both methods could produce reasonably comparable results.

RELATIVE AND ABSOLUTE NUTRIENT REQUIREMENTS

The nutrient requirements of fish are usually expressed as the percent protein content of the diet. The protein requirement of juvenile allogynogenetic crucian carp was found to be 39.3% by our study, which is higher than that of grass carp at 22.77 to 27.66% or 36.7%, that of Tilapia aurea at 36% (Davis and Stickney 1978), but comparable with that of black carp at 41% (Yang Guohua et al. 1985); mud carp, Cirrhinus molitorella, at 38.86% (Mao Yongqing et al. 1985b); rainbow trout at 40% (Satia 1974), and lower than that of snakehead, Channa micropeltes, at 52% (Wee and TAcon 1982); and plaice at 50% (Cowey et al. 1972).

The percentage of protein, however, is a ratio numerator whose value depends directly on the quantities of other components in the diet that make up the denominator (Bowen 1987). Moreover, the common expression of nutrient requirements solely in terms of a "dietary percentage" has itself limited value unless it is related to the food intake and subsequent growth of the animal (Tacon and Cowey 1985). For example, Ogino (1980) reported a decrease in the dietary protein requirement of juvenile carp and rainbow trout from 60 - 65% to 30 - 32% when the feeding level was increased from 2 to 4% body weight per day in both species.

Thus, the relative requirement expressed as dietary percentage is by no means a good measure of nutrient requirements except that it is convenient for formulating diet. The absolute requirement expressed as protein (gram) per (100 g) body weight per day not only accurately expresses the daily protein requirement by the fish but also allows valid comparison of data obtained by different laboratories. The relationship between relative protein requirement and absolute protein requirement could be expressed as:

Absolute protein requirement (g protein/100g body weight/day) = relative protein requirement (%) x feeding rate (g feed/100g body weight/day.

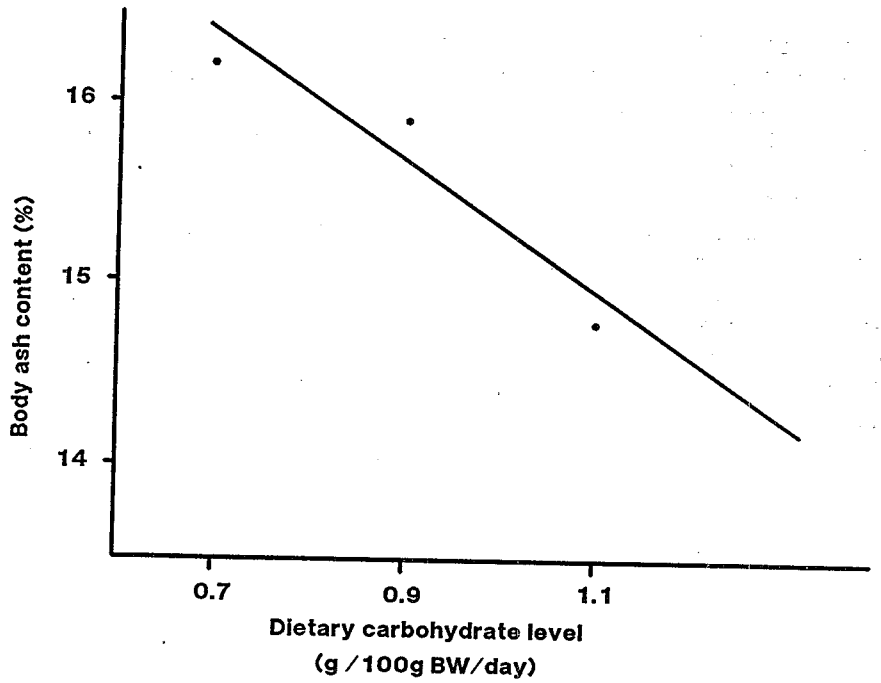

Figure 5. Relationships between dietary carbohydrate level and body ash content.

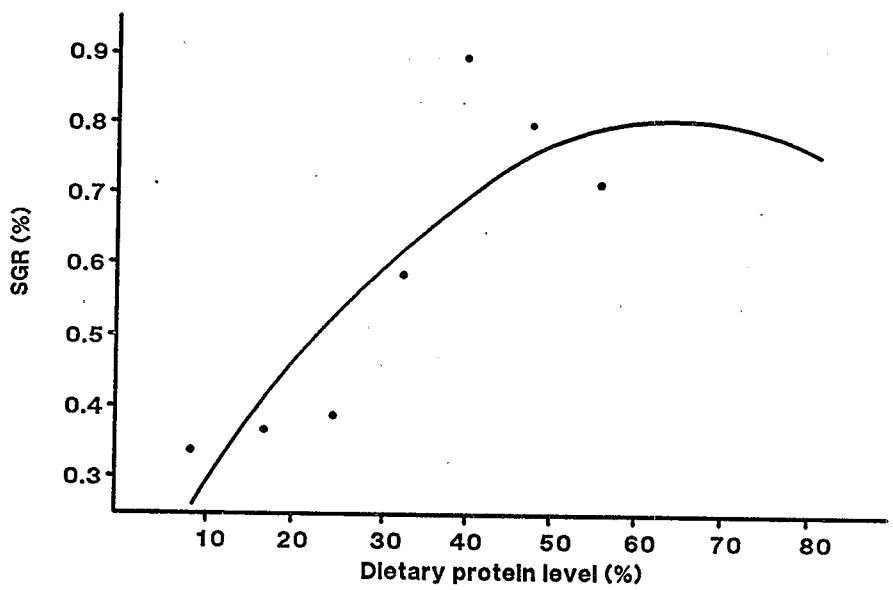

Figure 6. Effect of dietary protein level on the specific growth rate of juvenile gynogenetic crucian carp.

By contrast, if the relative protein requirement of fish is converted to the absolute protein requirement, the protein requirement of juvenile allogynogenetic crucian carp is found to be 1.3 g/100 g body weight/day by this study, which is higher than that of mud carp at 0.8 g/100 g body weight/day, that of snakehead at 1.04 g/100g body weight/day, that of plaice at 0.75g/100g body weight/day, comparable with black carp at 1.23 g/100 g body weight/day, and lower than that of grass carp at 1.65 to 1.76 g/100 g/100 g body weight/day, that of rainbow trout at 1.8 g/100 g body weight/day, that of Tilapia aureus at 3.18g/100g body weight/day. Obviously the comparison of protein requirement in different fishes in terms of absolutes requirement is quite different from that of the comparison of relative requirement between fishes. More data should be accumulated and further research is needed in this respect.

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POTENTIAL FOR USING CANOLA MEAL AND OIL IN FISH DIETS

by

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U.H.M. Fagerlund[1]

DEVELOPMENT OF CANOLA SEED

Canola seed and rapeseed share a common history as the former has been derived from the latter by genetical selection. The English word rape in rapeseed originates from the Latin word rapum, which means turnip. Both rape and turnip belong to the Brassica genus of crops, which also include mustard, cabbage, rutabagas, broccoli, and kale (Sauer and Kramer 1983). The oilseed forms of rape (rapeseed) have a long history; Indian Sanskrit writings dating from 3500 to 4000 years ago refer to rapeseed as a source of cooking and illumination oil (Boulter 1983). In Europe, rapeseed was employed for illumination and soap making before the thirteenth century (Bell 1982).

The impetus for Canadian rapeseed production stemmed from a critical need for rapeseed oil as a lubricant in naval and merchant ship steam engines during the Second World War (Miller 1988). Following the war, rapeseed oil was used increasingly as a food product until studies on rats given rapeseed oil caused human health concern because of the oil's high content of eicosenoic and erucic acids. This concern proved to be fallacious because there is no known hazard to humans from ingesting high erucic acid rapeseed oil, but it nevertheless stimulated Canadian plant breeders to develop cultivars of rapeseed that have low erucic acid content (Boulter 1983, Sauer and Kramer 1983). This initiative was enhanced further by additional observations of myocardial fat infiltration (cardiac lipidosis), myocardial necrosis (necrotic cardiac lesions), impaired mitochondrial function and enlarged pale adrenals in rats receiving high-erucic-acid rapeseed oil (Kramer and Sauer 1983, Sauer and Kramer 1983).

Although the development of rapeseed strains with low erucic acid content abolished cardiac lipidosis in rats, this was not the case with respect to myocardial lesions. Subsequently, however, it was shown that this latter problem was not a unique toxic consequence of ingestion of low erucic

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acid rapeseed oil but arose from an imbalance between primarily saturated and monounsaturated fatty acids (Kramer and Sauer 1983).

Besides high erucic acid content, most of the rapeseed produced in the world before 1974 was characterized by a high content of glucosinolate compounds. The hydrolysis of these compounds by the enzyme myrosinase in poorly processed meals or by intestinal microorganisms can yield several possible products (e.g., isothiocyanates, 5-vinyloxazolidine-2-thione or goitrin, thiocyanates, and nitriles) depending upon the conditions of hydrolysis (Tookey et al. 1980). Some of these, e.g., goitrin, suppress thyroid function. This is manifested by thyroid hypertrophy, reduced levels of intrathyroidal iodothyronines, depressed thyroid hormone titres and altered affinity of serum albumin for thyroxine (Higgs et al. 1979). Other consequences may include reduced conversion of thyroxine to 3,5,3'-triiodo-L-thyronine (T_3) in the liver and kidney and possibly alterations in the capacities and affinities of nuclear T_3 receptor sites. Concomitant depression of food intake, decreased food conversion, reduced growth, and histopathological changes in the liver and kidney were reported (Higgs et al. 1979).

These adverse responses naturally restricted the use of high glucosinolate rapeseed meal as a protein supplement in diets for poultry and livestock. Hence, genetical selection efforts were undertaken in Canada to produce rapeseed having low levels of erucic acid and glucosinolates. This was accomplished successfully in 1974 when the world's first Brassica napus variety of rapeseed with the foregoing traits was released. In 1977, this feat also was accomplished in a summer variety of Brassica campestris. The meals and oils derived from these new rapeseed varieties were shown subsequently to have dramatically improved nutritive value for poultry and livestock relative to the old varieties (Clandinin 1986). To clearly distinguish between the two types of oil-bearing seeds, the new varieties were renamed canola.

Today, Canada's production has switched almost entirely to canola seed. The oil from this seed, which contains less than 2% erucic acid versus 25% to 55% in high erucic acid content rapeseed oils (Ackman 1977), now accounts for 60% of all edible oil products such as mayonnaise, salad dressings, margarine and shortenings manufactured in Canada (Miller 1988). Canola meal, by definition, now contains less than 30 μmoles of glucosinolates per gram of air-dried, oil-free meal. The level of glucosinolates in canola meals is about eight- to ten-fold less than that in meals from high glucosinolate varieties of rapeseed (Clandinin 1986).

WORLD PRODUCTION OF RAPESEED/CANOLA SEED

The overall assessment of the potential for including novel sources of protein and lipid in animal diets includes considerations of supply, cost, and nutritive value. Of the 191.7 million tonnes of oilseeds produced on a worldwide basis in 1986/87, soybeans, cottonseed, rapeseed/canola seed, sunflower seed, and groundnuts (shelled) comprised, respectively, 51.4, 14.3, 10.3, 9.7, and 7.2 percent. Thus rapeseed/canola seed ranked third in the worldwide production of oilseeds.

The major producers of the 19.8 million tonnes of rapeseed/canola seed in that period were China (29.6%), Canada (19.2%), India (13.9%), Poland (6.5%), France (5.4%), West Germany (5.2%), the United Kingdom (4.8%), and Denmark (3.1%). Other nations such as Sweden, Czechoslovakia, East Germany, Hungary, Bangladesh and Pakistan each produced less than 500,000 tonnes (Canadian Gains Industry 1987). Thus, considerable rapeseed/canola protein and oil are available on a worldwide basis for inclusion in animal foods, considering that canola seed typically contains 41% to 43% fat and 21% to 23% crude protein (Robblee et al. 1986).

In Asia, however, most of the rapeseed grown is high in glucosinolate and erucic acid content. There the meal presently is used primarily as an organic fertilizer or a livestock feed supplement. Moreover, in the Asian countries of China, India, Pakistan, and Bangladesh, the oil is used domestically for human consumption (Pigden 1983). In Canada and Europe, canola/rapeseed meals are employed totally as high protein dietary supplements for animal foods (Downey 1983).

COST AND NUTRITIVE VALUE OF CANOLA/RAPESEED MEALS AS PROTEIN SUPPLEMENTS IN FISH FOOD

Per unit protein, the cost of fish meal is about twice that of canola meal and soybean meal in North America. Therefore, both of these oilseed meals are potentially very attractive as partial or total replacements for fish meal, when considering availability and cost only. Unlike soybean meal, however, it is not practical to consider canola and rapeseed meals as complete replacements for fish meal in circumstances where the fish species have high protein requirements and fish meal is providing most of the dietary protein needs. This is because canola/rapeseed products have about 37% protein compared with 65% to 72% for fish meal and 50% for soybean meal.

By contrast, this is not true for canola/rapeseed concentrates that are prepared from sound, cleaned, dehulled seeds from which oil- and water-soluble, nonprotein constituents have been removed (Jones 1979). These concentrates not only have extremely low glucosinolate content, but also higher protein and lower nitrogen-free extract (carbohydrate) levels than other oilseed protein meals (Table 1) and lupin, a potentially valuable legume for inclusion in fish foods (Hughes 1988).

Moreover, the fibre content in canola/rapeseed protein concentrates is reduced considerably below that of canola/rapeseed meals and other plant protein sources given in Table 1, except soybean meal. This is advantageous because fish cannot digest fibre and dietary levels exceeding 10% have been observed to depress growth, diet digestibility, food conversion, transit time of intestinal contents, and possibly mineral bioavailability in fish species (Higgs et al. 1988).

The reduced carbohydrate content in canola/rapeseed protein concentrate also should potentially enhance nutritive value relative to that of the canola/rapeseed meals in salmonid species. For instance, starch is digested poorly in salmonids especially when dietary levels are high. Moreover, starch

TABLE 1. PROXIMATE COMPOSITION AND MINERAL CONTENT OF SELECTED ANIMAL AND PLANT PROTEIN SOURCES

Proximate composition (% as fed)	Herring[1] meal	Anchovy[1] meal	Rapeseed[1] /canola meal	Rapeseed /canola protein concentrate	Soybean[1] meal	Cottonseed[1] meal	Sunflower[1] meal	Sweet[5] white lupin
Protein	72.0	65.5	37.0	63.0[3]	49.7	41.2	46.3	35.2
Crude lipid	8.4	4.1	1.7	8.0[3]	0.9	1.4	2.9	8.36
Fibre	0.7	1.0	12.0	4.7[3]	3.4	12.1	11.4	9.98
Nitrogen-free extract	0.4	6.6	33.5	14.8[3]	30.2	29.8	24.8	33.48
Ash	10.5	14.8	6.8	5.9[3]	5.8	6.5	7.6	2.88
Moisture	8.0	8.0	9.0	3.6[3]	10.0	9.0	7.0	10.1
Minerals: (g/kg as fed)								
Calcium	22	37.5	6.1	7.6[4]	2.6	1.7	4.1	4.1
Phosphorus	16.8	24.9	9.5	19.2[4]	6.3	11.0	9.1	4.8
Magnesium	1.5	2.5	5.5	7.3[4]	2.8	5.4	7.1	1.8
Manganese	0.006	0.011	0.054[2]	0.055[4]	0.037	0.021	0.019	0.85
Zinc	0.131	0.105	0.071[2]	0.121[4]	0.055	0.062	--	0.066
Copper	0.006	0.009	0.010[2]	--	0.020	0.020	0.004	0.007
Iron	0.125	0.218	0.159[2]	0.092[4]	0.133	0.208	0.031	0.081
Potassium	10.8	7.2	12.4	2.4[4]	20.7	13.9	10.6	6.9

[1] NRC (1983), [2] Clandinin et al. (1986), [3] J.D. Jones (1987, personal communication), [4] Bell et al. (1976) assuming 95% dry matter, [5] D. Sweere (1988, personal communication)

has a low net energy value for rainbow trout, and this is true even for glucose, which is known to be highly digestible (Hilton et al. 1987).

In regard to the mineral composition of the canola/rapeseed meals versus the fish meals, it is apparent that the former are lower in calcium, phosphorus, and zinc than the latter. When compared with soybean meal, however, canola meal is known to be a better source of available calcium, iron, manganese, phosphorus, and selenium for poultry and livestock (Clandinin 1986). Further, both canola meal and canola protein concentrate have a better balance of indispensable (essential) amino acids than is noted in all of the other oilseed meals and in the lupin included in Table 2 for comparison.

Here is should be mentioned that the nutritive value of animal and plant protein sources is governed to a great extent by how well the available levels and balance of ten indispensable amino acids derived from protein digestion conform to species needs at the sites of tissue protein synthesis. This can be examined by expressing each of the indispensable amino acids in a protein source, including cystine and tyrosine, as a percentage of the total weight of indispensable amino acids (g/100g protein) and then comparing these percentages to those derived from the indispensable amino acid requirements of, for example, rainbow trout and carp in the manner described by Oser (1959) for calculation of the essential amino acid index (EAAI).

This approach has proven to be very worthwhile for predicting the performance of carp (Murai et al. 1984) and it assumes an adequate dietary energy intake as well as a balanced mixture of indispensable and dispensable amino acids. Although the indispensable amino acid pattern in fish muscle and whole body protein shows the best correspondence to the requirements for carp and rainbow trout by this procedure (EAAI=97), it also is apparent from Table 2 that the indispensable amino acid balance in canola meal shows excellent agreement as well (EAAI=95). Therefore, canola/rapeseed meals and concentrates have potentially high nutritive value for finfish if complete availability of the indispensable amino acids is assumed.

Data on average and apparent protein digestibility coefficients for canola and rapeseed meals in rainbow trout provided in Table 3, however, show that rapeseed meal has less available protein than canola meal, full-fat cooked soybeans, soybean meal, or fish meal. Moreover, canola meal has lower available protein than soybean meal in this species. Also, in Atlantic salmon, the availability of canola protein is lower than that of soybean meal and fish meal but differences between the protein digestibility coefficients for these protein sources are less when the fish are in seawater. A trend similar to that for protein was observed for the availability of energy from these protein sources in trout and salmon (Table 4).

The causes of the reduced protein and energy digestibility of canola/rapeseed meals probably relate to their higher content of fibre compared to that in soybean meal, and to their levels of phytic acid, tannins, and glucosinolates. Phytic acid, the hexaphosphate of myoinositol, can decrease protein and mineral bioavailability (e.g., zinc) by mechanisms described by Higgs et al. (1988). It is clear that there is a need to remove this anti-nutritional factor by novel processing procedures. Tannins also are known

TABLE 2. RELATIVE PROPORTIONS OF ESSENTIAL AMINO ACIDS (EAA) IN SELECTED ANIMAL AND PLANT PROTEIN SOURCES IN RELATION TO MEAN EAA REQUIREMENTS (EXPRESSED AS % OF TOTAL EAA) OF RAINBOW TROUT AND CARP (OGINO 1980, TACON 1985). EAA INDEX FOR EACH PROTEIN SOURCE WAS CALCULATED ACCORDING TO OSER (1959) USING THE PATTERN OF AMINO ACID REQUIREMENTS OF CARP AND RAINBOW TROUT AS THE REFERENCE STANDARD

Protein source/ requirement	Source[a]	Arg	His	Ils	Leu	Lys	Met + Cys	Phe + Tyr	Thr	Trp	Val	EAA Index	Major limiting amino acids
Fish whole body protein	1 (ISD)	12.1 (1.0)	5.26 (0.74)	7.98 (0.33)	14.7 (0.52)	16.8 (0.42)	7.70 (1.0)	14.9 (0.47)	9.29 (0.36)	1.92 (0.52)	9.38 (0.93)	97	THR
Fish muscle	2	11.2	5.78	8.06	14.8	17.0	8.12	15.2	8.85	2.29	9.34	97	THR
Whole herring meal	3	13.0	4.62	8.78	14.6	15.0	7.92	13.8	8.14	2.16	12.1	94	THR
Anchovy meal	3	11.4	4.86	9.36	15.1	15.2	7.81	15.2	8.33	2.25	10.6	96	THR
Canola meal	4	13.1	6.01	7.41	14.9	12.8	9.87	14.0	9.74	2.56	9.52	95	LYS
Canola protein concentrate	5,6	12.8	6.89	8.31	14.6	11.6	8.49	14.4	9.54	3.41	9.96	94	LYS
Soybean meal	3	14.7	4.87	9.84	14.9	12.7	5.84	16.5	7.75	2.76	10.2	91	METH, CYS, THR, LYS
Cottonseed meal	3	23.8	5.62	6.82	12.3	9.49	5.94	17.2	6.89	2.67	9.33	86	LYS, THR, MET+CYS
Sunflower meal	3	18.1	5.04	9.20	15.7	7.86	7.76	15.3	7.90	2.50	10.6	89	LYS, THR
Lupin seed meal	7	22.3	3.52	7.65	15.3	9.67	5.44	19.8	7.98	1.47	6.89	82	LYS, MET+CYS VAL, THR, HIS
Rainbow trout & carp EAA requirements	8	11.6	4.75	7.45	13.5	16.8	8.08	16.0	10.6	1.74	9.51		

[a](1) Mean of values for coho (Arai 1981), masu salmon (Ogata et al. 1983), chum salmon (Akiyama et al. 1985), channel catfish (Wilson and Poe 1985a), rainbow and Atlantic Salmon (Wilson and Cowey 1985), goldfish, golden shiner and fathead minnow (Gatlin 1987) and chinook salmon (Dosanjh et al. 1988, unpublished), (2) Cho et al. (1985), (3) NRC (1981), (4) Clandinin et al. (1986), (5) Bell et al. (1976), (6) Jones (1979), (7) de la Higuera et al. (1988), (8) Ogino (1980).

TABLE 3. AVERAGE PERCENTAGE APPARENT PROTEIN DIGESTIBILITY COEFFICIENTS OF SELECTED DIET INGREDIENTS MEASURED IN RAINBOW TROUT, ATLANTIC SALMON, CHANNEL CATFISH AND TILAPIA

Ingredient	International feed number	Rainbow trout	Atlantic salmon FW[a]	Atlantic salmon SW[a]	Channel catfish	Tilapia	Source[b]
Fish meal (sardine)			---	---		86	1
Herring meal	5-02-000	92	90.2	94.0			2,3
		86.7					5
Anchovy meal	5-01-985	85.2	---	---	85-90		5,7
Soybean meal	5-04-604					91	1
	5-04-612	96	89.4	90.0	93.0-97.0		2,3,6
Soybeans, full fat grnd, oven roasted							
127°C, 10 min.		40.3					5
204°C, 10 min.		78.2					5
Soybean, full fat cooked	5-04-597	96					3
Canola meal	5-03-871	83.2-87.1	80.8	86.3			2,4
Rapeseed meal	5-03-871	77	---	---			3
		76.4					5
Cottonseed meal	5-01-621		---	---	80-83		6
	5-07-874	76-78	---	---			5
Lupin		85.2					8

[a] FW = Freshwater, SW = Seawater. [b] (1) Hanley (1987), (2) Lall (1988, unpublished data), (3) Cho et al. (1982), (4) Hilton and Slinger (1986), (5) Smith et al. (1980), (6) Wilson and Poe (1985b), (7) Robinson and Wilson (1985), (8) Hughes and Smith (1988).

TABLE 4. AVERAGE PERCENTAGE APPARENT DIGESTIBILE ENERGY COEFFICIENTS OF SELECTED DIET INGREDIENTS MEASURED IN RAINBOW TROUT, ATLANTIC SALMON, CHANNEL CATFISH AND TILAPIA

Ingredient	International feed number	Rainbow trout	Atlantic salmon FW[a]	Atlantic salmon SW[a]	Channel catfish	Tilapia	Source[b]
Fish meal (sardine)			---	---		79.8	1
Herring meal	5-02-000	91	93.2	94.7			2,3
		91.5					4
Anchovy meal	5-01-985	91.3					4
Soybean meal	5-04-604					56.6	1
	5-04-612	75	76.0	77.9	73		2,3,5
Soybeans, full fat grnd, oven roasted							
127°C, 10 min.		51.3					4
204°C, 10 min.		77.3					4
Soybean, full fat cooked	5-04-597	85					3
Canola meal	5-03-871		62.9	72.7			2
Rapeseed meal	5-03-871	45					3
		65.2					4
Cottonseed meal	5-01-621				65-80		5
	5-07-874	58-64					4
Lupin		62-66					6

[a] FW = Freshwater, SW = Seawater. [b] (1) Hanley (1987), (2) Lall (1988, unpublished data), (3) Cho et al. (1982), (4) Smith et al. (1980), (5) Wilson and Poe (1985b), (6) Hughes and Smith (1988).

TABLE 5. ACCEPTABLE (BASED ON GROWTH AND FOOD CONVERSION RESPONSES) DIETARY LEVELS OF RAPESEED/CANOLA PRODUCTS FOR SELECTED FISH SPECIES

Species	Initial size (g)	Duration (Days)	Rapeseed/ canola product	Dietary glucosinolate content (µg 3 butenyl isothiocyanate/g)	Acceptable dietary level (% dry wt)	Acceptable dietary level (% of dietary protein)	Source[c]
1) Rainbow trout (*Salmo gairdneri*)	4-5	161	Rapeseed meal	223	22.1	21.4	1
			Rapeseed flour	223	<22.1	<26.7	
			Rapeseed protein concentrate	100	>22.1	>28.8	
S. gairdneri	105	108	Canola meal	340	20[a]	18.0[a]	2
S. gairdneri	2	112	Canola meal	158	<13.5[a]	<13.3[a]	3
	2.2	112	Canola meal with/without supplemental arginine, lysine, zinc or energy (lipid)	441	<35[a]	<35[a]	
S. gairdneri	juvenile	84	Canola meal	164	<26.6[a]	<25.0[a]	4
2) Coho salmon (*Oncorhynchus kisutch*)	4-4.5	168	Canola meal	366.8	22	17.8	5
3) Chinook salmon (*O. tshawytscha*)	0.15-0.18 (dry wt)	83	Canola meal (Candle)	113	17.1	13.0	6
			Canola meal (Tower)	89	16.0	13.4	
			Rapeseed protein concentrate	0	>19.9	>24.4	
O. tshawytscha	1	84	Canola meal (Altex)	300	23.2	23	7
4) Common carp (*Cyprinus carpio*)	2.5	74	Rapeseed meal (low glucosinolate; toasted)	75	27.7[a]	28[a]	8
			Rapeseed meal (high glucosinolate; toasted)	2580	27.7[a]	28.4[a]	
5) Tilapia (*Sarotherodon mossambicus*)	12.4-13.3	63	Rapeseed meal (high glucosinolate)	3130	41.3	49.4	9
(*mossambicus* X *aurea* hybrids)	0.32	57	Canola meal	114	31.6[b]	38.9[b]	10

[a]Estimated [b]Growth not depressed but slight decline in food conversion

[c](1) Yurkowski et al. (1978), (2) Hardy and Sullivan (1983), (3) Hilton and Slinger (1986), (4) Leatherland et al. (1987), (5) Higgs et al. (1979), (6) Higgs et al. (1982), (7) Higgs et al. (1983), (8) Dabrowski and Kozlowska (1981), (9) Jackson et al. (1982), (10) Higgs et al. (unpublished data).

to impair protein availability (Sosulki 1979), although their effect on the nutritive value of rapeseed/canola proteins may be less than that of phytic acid and the glucosinolates.

Information regarding the nutritive value of rapeseed and canola meals, flours, and concentrates for various finfish species is compiled in Table 5. The acceptable dietary levels given were based on equivalent growth and food conversion responses between test and control fish. For rainbow trout, there appears to be considerable controversy regarding acceptable dietary levels of rapeseed or canola meals irrespective of whether this is judged on the basis of percentage of dietary dry weight or protein. This may reflect differences between studies in the initial fish size (age), level of dietary (glucosinolate) intake on a per body weight basis, and cultural conditions.

Rapeseed protein concentrate was noted to be more acceptable to rainbow trout (Yurkowski et al. 1978) and to chinook salmon (Higgs et al. 1982) than rapeseed and canola meal, respectively. Generally, the nutritive value of canola meal for Pacific salmon species is consistently good, and acceptable dietary levels range from 13 to 23% of the dietary protein. For warmwater species, the nutritive value of rapeseed meals of low or high glucosinolate content and of canola meal appears to be higher than noted for coldwater species. For instance, in one study on tilapia (Jackson et al. 1982), close to 50% of the dietary protein as high glucosinolate rapeseed meal was found to be acceptable.

Three studies conducted on salmonids (Higgs et al. 1983, Leatherland et al. 1987, Fagerlund et al. 1987) provide conclusive evidence that glucosinolates are mainly responsible for depression of growth and protein utilization when the diets contain high levels of canola meal. In this situation, concurrent supplementation of the diets with T_3 reinstates normal growth. In coho salmon, doses of 1.2 to 10 ppm of T_3 are effective, and dietary protein quality is not compromised until canola meal exceeds about 27% of the dietary protein (Figure 1). Interestingly, hypertrophy of the thyroid follicle epithelial cells was still noted in juvenile coho salmon as the dietary canola meal levels were increased even though T_3 was given orally at 9.45 ppm. Moreover, dietary protein levels also influenced the response (Figure 2).

The foregoing work serves to illustrate the importance of totally eliminating the glucosinolates from canola meal through further genetical selection and processing innovations. Significant progress is being made in both areas and one processing method developed by Rubin and Diosady (1984) and Naczk et al. (1986) not only almost entirely eliminates glucosinolates from rapeseed or canola meals, but also concurrently elevates the protein content to about 50% because of fibre, carbohydrate and lipid reduction.

Thus the rapeseed and canola meals have high nutritive value for warmwater species. The use of canola meal as a protein supplement in diets for coldwater species, especially rainbow trout, however, requires further enhancement of its nutritive value through reduction of fibre, carbohydrate, phytic acid and glucosinolate content.

AVAILABILITY, COST AND NUTRITIVE VALUE OF RAPESEED AND CANOLA OIL FOR FINFISH SPECIES

In 1986/87, rapeseed/canola oils ranked third (6.79 million tonnes) in terms of the world production of oils and fats. The production of rapeseed/canola oils was exceeded only by palm (7.87 million tonnes) and soybean oils (14.5 million tonnes) (Canadian Grains Industry 1987).

Generally, the prices of fish oils are less than those of edible vegetable oils such as soybean and rapeseed/canola oils and animal fats such

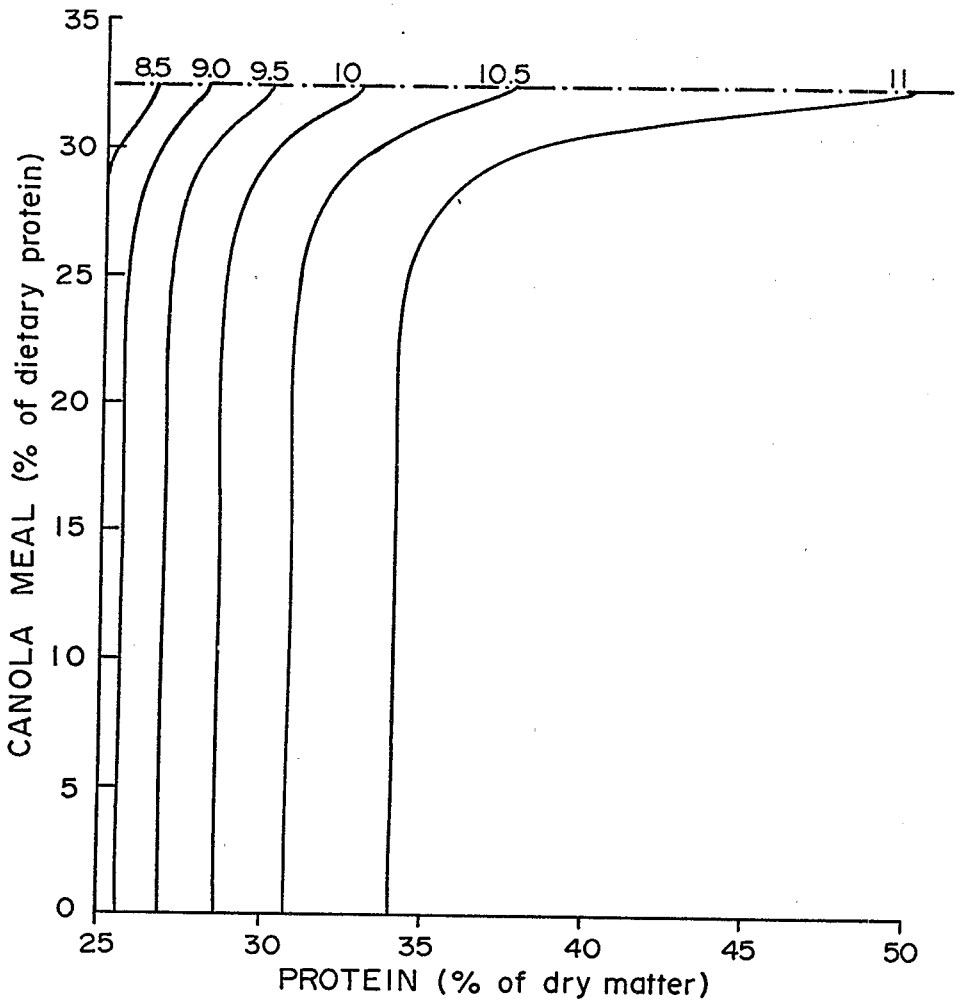

Figure 1. Influence of dietary level of canola meal and protein on final mean weight of juvenile coho salmon (T_3 = 10 ppm) (Higgs et al., unpublished data).

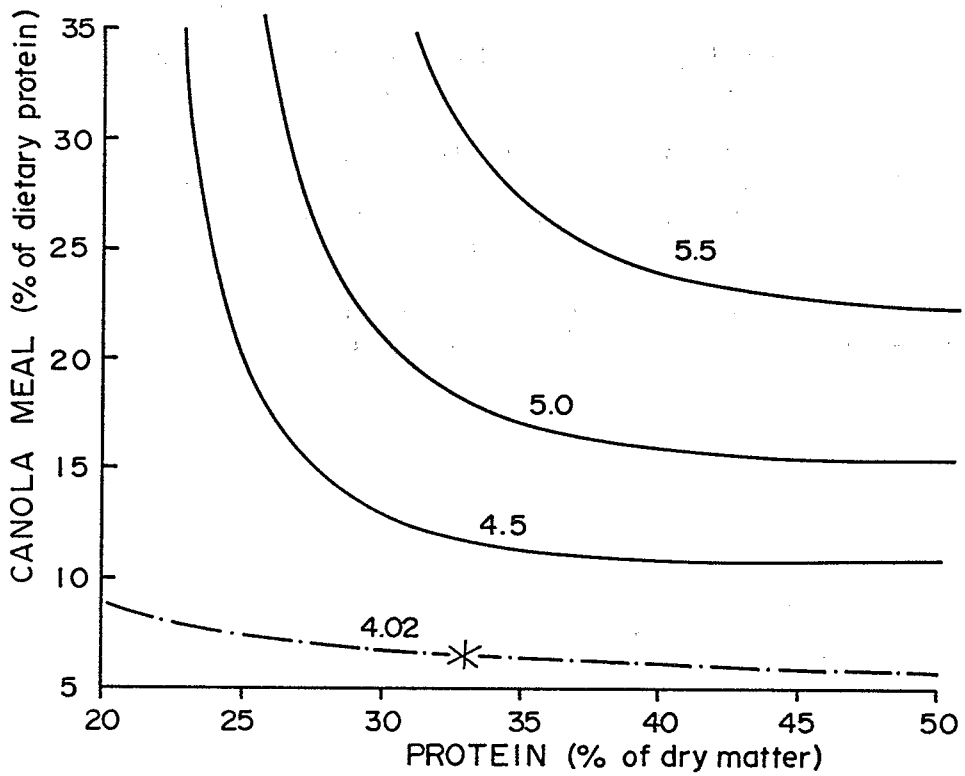

Figure 2. Influence of dietary level of canola meal and protein on thyroid follicle epithelial cell height (μm; T_3 = 9.45 ppm) of juvenile coho salmon (Higgs et al., unpublished data).

as lard (Bimbo 1987). As Dosanjh et al. (1988) point out, however, it is likely that within 10 years, the cost and availability of high quality marine lipids for fish diets will be much higher and lower, respectively, than at present because of increased utilization of these valuable lipids in the human diet because of health concerns.

Thus, it may be prudent to identify alternate sources of supplemental lipid to marine lipids for inclusion in fish diets in the near future. The selection of the appropriate alternate lipid source for a given species depends upon its needs for essential fatty acids. In this regard, the essential fatty acid requirements of some of the important cultured finfish species are provided in Table 6.

Here, it is readily apparent that considerable differences exist between species in their essential fatty acid requirements. Chum salmon, carp, and eel perform well on balanced amounts (equal amounts) of linolenic acid (C18:3ω3) and linoleic acid (C18:2ω6). By contrast, tilapia requires solely C18:2ω6 or C20:4ω6 (arachidonic acid) and rainbow trout and coho salmon have more stringent needs for fatty acids of the linolenic series (ω3 fatty acids). Further, juvenile fall chinook salmon appear to metabolically adapt to high dietary ω6 fatty acid content (2.6%; Dosanjh et al. 1988) when the ω3 fatty

TABLE 6. ESSENTIAL FATTY ACID REQUIREMENTS OF SELECTED FISH SPECIES

Species	Source[a]	Essential fatty acid requirements
Rainbow trout (Salmo gairdneri)	1	≥ 20% and ≤ 80% of the dietary lipid level (>5%) as linolenic acid (C18:3ω3) or ≥ 10% and ≤ 40% as a combination of eicosapentaenoic acid (C20:5ω3) and docosahexaenoic acid (C22:6ω3). Growth is inhibited when C18:2ω6 > 1%.
Chum salmon (Oncorhynchus keta)	1,2	10% of the dietary lipid content as a combination of C20:5ω3 and C22:6ω3 (ω3 HUFAs). 1% C18:2ω6 (linoleic acid) + 1% C18:3ω3.
Coho salmon (Oncorhynchus kisutch)	3	1-2.5% of the diet as C18:3ω3 Growth is inhibited when C18:2ω6 > 1% or when C18:3ω3 > 40% of dietary lipid content.
Carp (Cyprinus carpio)	1	1% C18:2ω6 + 1% C18:3ω3
Eel (Anguilla japonica)	1	0.5% C18:2ω6 + 0.5% C18:3ω3
Tilapia (Tilapia zillii)	1	1% C18:2ω6 OR 1% C20:4ω6

[a] (1) Watanabe (1982), (2) Takeuchi and Watanabe (1982), (3) Yu and Sinnhuber (1979)

acid content is simultaneously high. Rainbow trout and coho salmon show reduced growth, however, when the dietary level of C18:2ω6 exceeds 1%.

The percentages of some of the important fatty acids in canola and rapeseed oil, selected marine lipids, and soybean and corn oil are given in Table 7. The most striking difference in fatty acid composition between the edible vegetable oils and the marine lipids is the total absence of ω3 highly unsaturated fatty acids (ω3HUFAs; C20:5ω3+C22:6ω3) in the former lipids. The ω3HUFAs have higher essential fatty acid activity than C18:3ω3 in trout and their presence is essential in the diets of some cultured marine fish species (Watanabe 1982). Relative to soybean oil, canola oil has more C18:1ω9 (oleic acid) but less C18:2ω6. The percentages of C18:3ω3 in these sources are about equivalent (9%). Corn oil, another commonly used lipid source in purified fish diets, is almost devoid of linolenic acid and is very high in linoleic acid.

In relation to the finfish fatty acid requirements given in Table 6, canola oil would seem to have the best balance between linoleic and linolenic acids to satisfy the needs of chum salmon, carp, eel, and tilapia. Moreover, in species that are sensitive to high dietary levels of linoleic acid (e.g., rainbow trout and coho salmon), this should prove to be a better source of supplemental dietary lipid than either corn or soybean oil.

Several studies conducted on salmonids have shown that canola oil is an excellent alternative supplemental dietary lipid (Cho et al. 1974, Dosanjh et al. 1984, Dosanjh et al. 1988). By contrast, an unpublished study on rainbow trout, (R.S. Parker and J.D. Hendricks) reported by Dosanjh et al. (1984), found pathological changes, especially in the heart after feeding diets with a high erucic acid content. It should be mentioned, however, that the fatty acid profile of canola oil containing low erucic acid content has not been found to induce pathology in Pacific salmon (Dosanjh et al. 1984). Further, complete substitution of canola oil for corn oil in tilapia diets did not compromise either growth or food conversion (Figure 3).

Therefore, the potential for including canola oil in cultured finfish diets as a source of supplemental lipid appears to be excellent although studies of long duration are required to confirm the preliminary findings and ensure the absence of pathological changes.

TABLE 7. SOME IMPORTANT FATTY ACIDS IN SELECTED MARINE AND EDIBLE VEGETABLE OILS (g/100g)

Fatty Acid	Pacific[1] herring oil	Chilean anchovetta[2] oil (Talcahuano)	Rapeseed oil[3] (Echo; high erucic, Canada)	Canola[1] oil	Soybean[3] oil	Corn[3] oil
16:0	13.3	16.5	2.5	4.4	15.3	11.5
18:0	1.8	2.8	1.0	1.7	4.2	2.2
18:1ω9	14.1	8.4	32.5	58.3	23.6	26.6
18:2ω6	2.9	1.1	18.8	21.7	48.2	58.7
18:3ω3	1.4	0.4	8.9	9.4	8.7	0.8
20:1ω9	5.3	8.3	12.0	1.6	---	---
20:5ω3	9.0	14.5	---	---	---	---
22:1ω9	17.1	5.7	23.5	0.8	---	---
22:6ω3	9.6	8.9	---	---	---	---
ω6/ω3	0.12	0.048	2.1	2.3	5.5	73.4
ω3HUFAs (20:5ω3+22:6ω3)	18.6	23.4	---	---	---	---
Unsat./Sat	4.9	2.3	23.4	15.0	4.1	6.2

[1] Dosanjh et al. (1988)
[2] Ackman (1982)
[3] Downey (1983)

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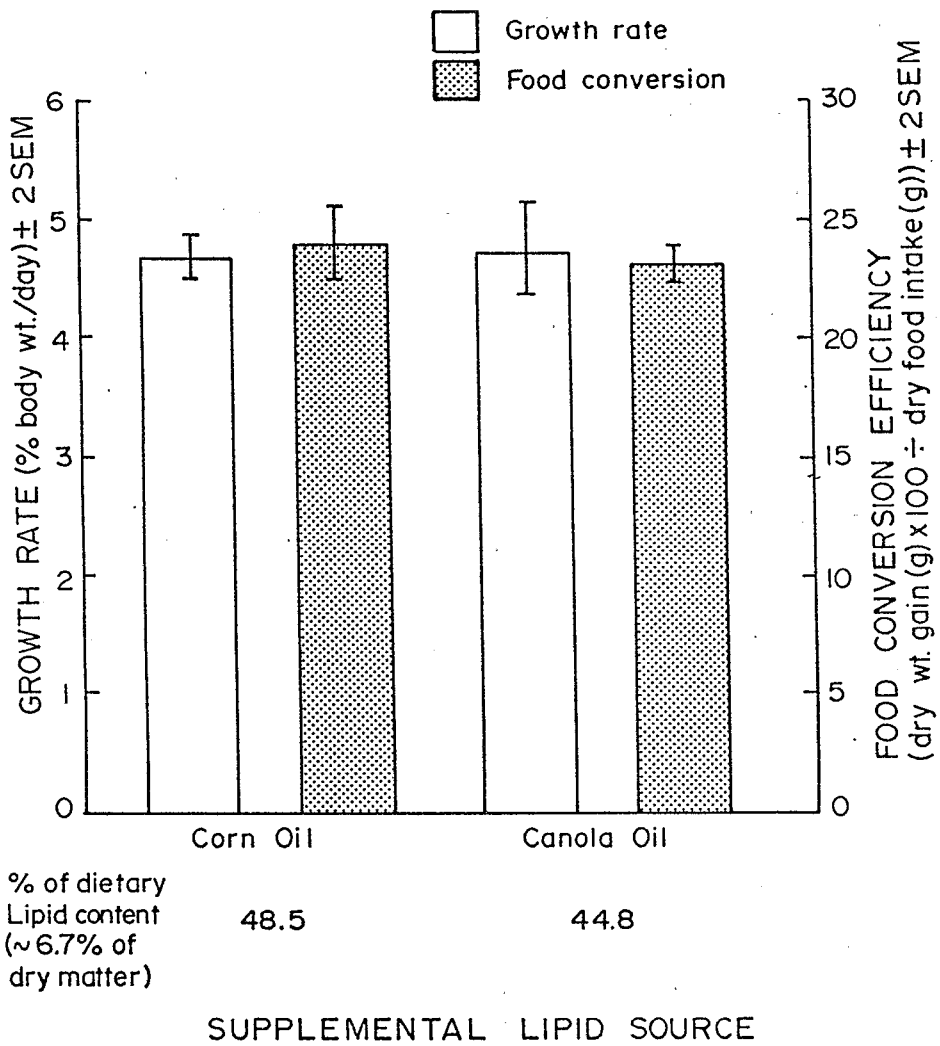

Figure 3. Growth rate and food conversion of Tilapia mossambicus x aurea hybrids in relation to source of supplemental dietary lipid. Fish were kept in recirculated 27-29°C filtered dechlorinated city water and fed 6 to 8X daily their prescribed diet to near maximum ration for 57 days (Higgs et al., unpublished data).

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MOBILIZATION OF BODY RESERVES DURING INDUCED GONADAL DEVELOPMENT IN THE JAPANESE EEL, *ANGUILLA JAPONICA* (TEMMINCK AND SCHLEGEL): THE ROLE OF THE PITUITARY GLAND AND CORPUSCLES OF STANNIUS

D.K.O. Chan[1] and E.Y.L. Lau[1]

INTRODUCTION

The eel is a terminal breeder. At the beginning of its seaward spawning migration, the animal stops feeding altogether while the gonad is still very small and immature. The animal, therefore, must rely on the mobilization of body reserves for both gonadal development and for the long journey to the spawning ground thousands of miles away. The first successful induction of gonadal development, spawning and hatching was achieved by Yamamoto et al. (1974a, b) followed by a group of Chinese researchers in Xiamen in 1978 and Wang et al. (1980). Induction of oocyte maturation and fertilization in vitro also has been reported (Yamauchi and Yamamoto 1982). Up to now, all hatched eels died at the prelarval stage, presumably due to insufficient yolk in the eggs to carry embryonic development to the larvae stage when feeding would begin. Furthermore, the maturing eels were very fragile and many died before reaching full sexual maturity (Ochiai et al. 1974, Yamauchi and Yamamoto 1982). The present study was conducted to evaluate the status of the biochemical system(s) responsible for intermediary metabolism and protein/purine catabolism as the gonads developed and to determine the endogenous source of the nutrients required for gonadal growth. Concomittant changes in the pituitary and corpuscles of Stannius also were observed.

MATERIALS AND METHODS

EELS

A total of 86 male and 89 female eels were used over a period of 5 years. They were caught during their spawning migration down the Pearl River estuary in late autumn. In the laboratory, the eels were transferred to sea water at 20 to 22°C and maintained without food. The water quality was controlled by circulating charcoal filters and flow-through UV-sterilizers.

TREATMENT

Eels were injected intraperitoneally once weekly without anaesthesia, minimizing stress by raising the net gently and injecting under water.

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Control eels were given saline. Experimental eels received 1 i.u./g HCG (Sigma) and 2 μg/g acetone-dried powder of mature carp pituitary extracted into saline and centrifuged at 5000 rpm for 5 minutes. Some eels received 3 priming doses of 1 μg/g oestradiol-17ß per week.

SAMPLE ANALYSES

Controls and homone-treated eels were sacrificed from 3 to 32 weeks by spinal cord transection. After taking a blood sample by cardiac puncture and flushing the tissues clear of blood with heparinized saline, the gonad, liver and gut were taken and weighed. Gonads and Stannius corpuscles were fixed in Bouin and the pituitary gland in Bouin-Hollande for histochemical and morphometric study. Samples of liver, white epaxial muscle, and red muscle were taken for water and lipid content by drying and defatting with petroleium ether and, later, were extracted with 0.1M HNO_3-10% acetic acid for Ca and phosphate determination. Portions of liver and white epaxial muscle were homogenized in 20-times its weight of ice-cold buffer (10 mMTris,pH7.5,1mMß-mercaptoethanol and 1mMEDTA-Na_2) in a Potter-Elvehjem homogenizer and a Teflon pestle and run at 10,000 rpm for 1 minute, sonicated for 5 minutes and centrifuged at 108,000 x g at 4°C for 30 minutes. The supernatant was used for enzyme determination at a thermostated temperature of 20 ± 0.5°C. Conditions (pH, substrate and cofactor concentrations) for enzyme reaction rates were pre-determined for each enzyme to yield optimal rates (cf. Bergmeyer 1974). Blood glucose, lactate, pyruvate, tissue glycogen, plasma total lipid, free acid, protein, amino acid, phosphate and ammonia were determined as in Chan and Woo (1978). The carcass was then stored frozen at 20°C. After thawing some months later, a 1-cm-thick segment of the truck at the level of anterior tip of the dorsal fin was taken and divided according to the skin, red muscle, white muscle and vertebra; the size of each compartment was determined gravimetrically. The vertebra then was extracted with an acid mixture for Ca and P determination. Ca was determined by atomic absorption spectrophotometry.

RESULTS

GONADS

Male eels responded well to HCG alone. The gonadosomatic index reached >40% in some cases by 18 to 20 weeks of treatment. Milt could be obtained by stripping and several animals spermiated spontaneously. Mature male eels appeared black with marked enlargement of the eye and shortening of the jaws. Male eels were divided into groups according to the condition of the testis showing (I) spermatogonia, (II) spermatocytes, (III) spermatids and maturing sperms, (IV) fully mature testis with only sperms, and (V) regressed testis with sperm discharged (Figure 1). Only a small percentage of male eels failed to respond to gonadotropin treatment and these had a GSI of <0.01%.

Female eels responded slowly to HCG treatment alone but the response accelerated if carp pituitary extract was added. About 40% of the eel did not respond to the treatment, their ovaries remained small (GSI of 3 to 5% at 30

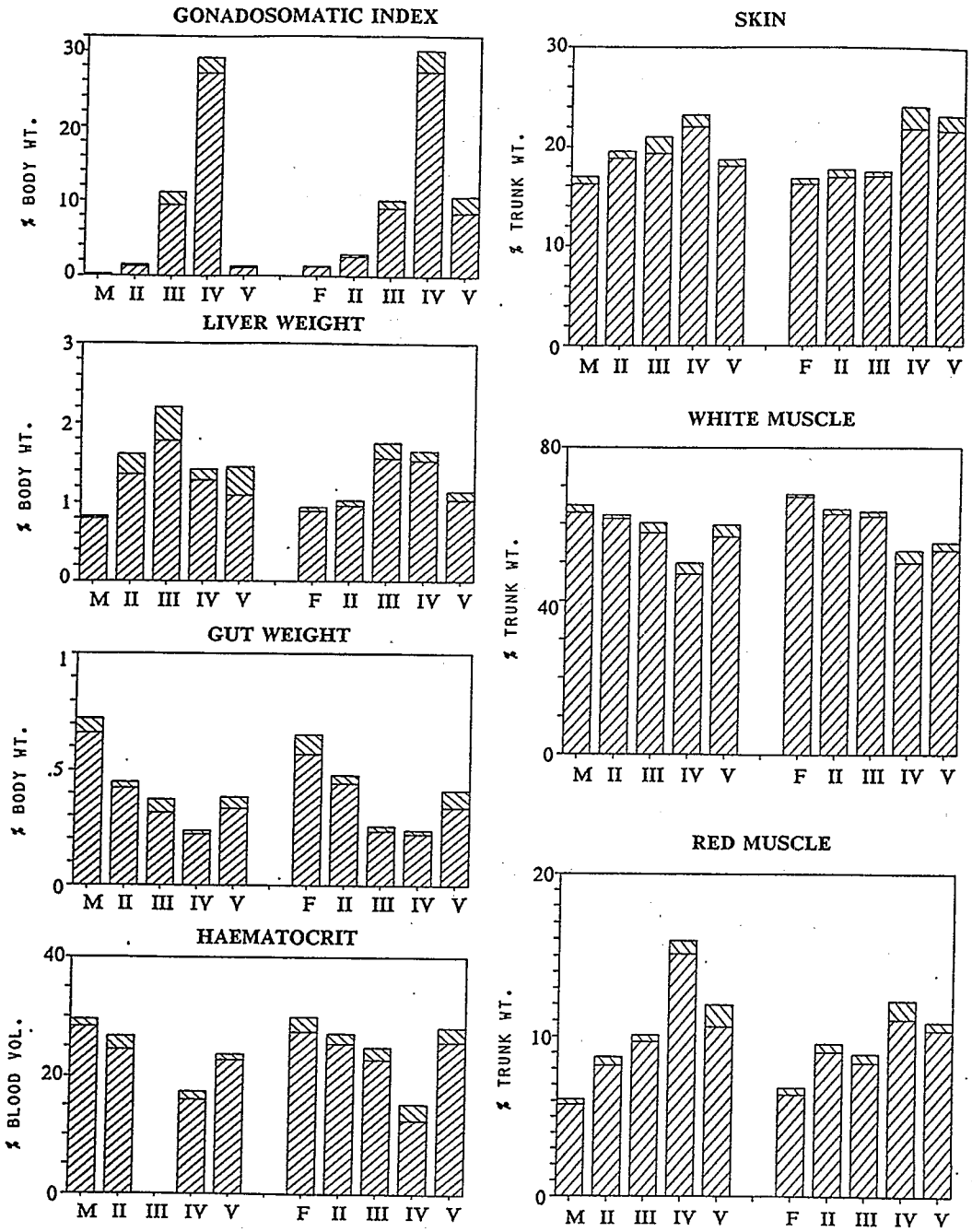

Figure 1. Changes in gonadosomatic index, liver and gut weight, haematocrit and proportion of skin/white muscle/red muscle in the trunk region of male and female eels during induced gonadal development (M=male control, F=female control; II, III, IV, V= gonadal stages) (mean+SEM).

weeks) (data omitted here). Of those that responded to the treatment, 4 animals reached GSI >60% by 20 to 25 weeks and these were invariably large animals weighing about 700 to 900g. These were not sampled until after regression set in at about 30 weeks. Priming with oestrogen also appeared to be beneficial, especially for the initiation of vitellogenin synthesis in the liver but it was not essential. In male eels, oestrogen markedly blocked testis development at the spermatocyte/spermatid state (n=5, not shown in figure). As it was not possible to distinguish between the sexes at the early stages, oestrogen was discontinued in later experiments. When the GSI >30%, some of the female eels took on a greyish silver color with the tip of the snout and fins turning black. Eye enlargement was noted. They were divided into groups showing (I) ovary with oogonia, (II) endogenous vitellogenesis with lipid vesicles, (III) exgenous vitellogenesis with accumulation of yolk platelets, (IV) maturing ovary with oocytes full of yolk granules, and (V) atretic ovary. Mature oocytes measured about 1 mm in diameter.

PITUATARY GLAND

The somatotrophs (Luxol fast blue acidophil, occupying the center of the cell chords) dominated the mesoadenohypophysis in control eels. As the gonads developed, gonadotrophs hypertrophied (basophils: aldehyde fuchsin and PAS, occupying the periphery). The remaining somatotrophs were degranulated with small cytoplasmic volume and nuclei. This change began within 3 weeks after hormonal treatment and was pronounced as GSI >10% body weight. Differentiation of gonadotrophs was absent in eels that did not respond or responded slowly (GSI about 3-5% in 30 weeks) to the exogenous homone. In the proadenohypophysis, as the eels were maintained in seawater, the prolactin cells were regressed in the control eels compared with eels maintained in freshwater. Regression went further with gonadal development in both sexes so that fewer prolactin follicles could be seen in the pituitary as GSI >20% body weight. The thyrotrophs were few and triangular in shape in control eels and showed stimulation beginning at the early stages of gonadal development (stage II). As GSI >20%, they dominated the proadenohypophysis and the cells became round, with large nuclei, and were loaded with glycoprotein granules.

CORPUSCLES OF STANNIUS

In starved control eels, the cells in the Stannius corpuscles were uniform in appearance and loaded with large dense granules (aldehyde fuchsin or PAS). As the gonads responded to hormonal treatment, the corpuscles showed marked hypertrophy and hyperplasia in both sexes. Many large cells became degranulate especially in large corpucles, and many smaller cells located near the lumen contained varying amounts of AF/PAS granules. In male eels, the response began at early spermatogenesis. In female eels, the effect developed slowly and peaked as exogenous vitellogenesis became complete. In both sexes, the corpuscles began to re-accumulate granules following gonadal regression. Estrogen alone had limited effects on ovarian development but suppressed the testis. Estrogen failed to affect the corpuscles although plasma Ca was raised markedly by this treatment, especially in the male eel.

BODY COMPARTMENT SIZES

The haematocrit values were maintained during 30 weeks of starvation but declined by 60% as the gonads developed beyond GSI of 20% (Figure 1). In eels with GSI >40%, the haematocrit became so low in some cases that the animal showed signs of respiratory stress (hyperventilation, air-gulping) even when ambient oxygen was close to air-saturation.

The liver size incresed significantly (up 50%) in both sexes as the gonads developed. The gut weight remained unaltered up to 30 weeks of starvation but shrunk and lost more than half its weight as GSI >20%. In the typical cross-section of the trunk region, the proportion of skin and red muscle increased at the expense of the white muscle compartment. Loss of white muscle drastically altered the shape of the tail, which became flat and thin.

CALCIUM AND PHOSPHORUS METABOLISM

In female eels, plasma Ca and P rose sharply as exogenous vitellogenesis was initiated, showing a 10- to 15-fold increase at the peak. In the treated male eel, the plasma Ca and P rose slightly during the early phase of spermatogenesis (stage II) but levelled off later. The amount of calcium deposited in the ovary was 10 times that of the testis of equivalent weight. Loss of Ca and P from the vertebrae was detected as GSI >10% and progressed at similar rates in both sexes as the gonads enlarged. The rate of P removal from the bone was more rapid, esppecially in the female, so that the ratio of Ca/P in the bone increased significantly from 1.8 to 2.0 as the GSI >20%.

In male eels, the white muscles began to show Ca deposition as GSI >10%. At maturity, the amount of Ca in white muscle was more than 8-fold that of the corresponding starved controls and the values returned to control levels after sperm discharge. In female eels, this Ca deposition occurred much later when the ovary >20% body weight. Eels that showed little response to HCG treatment showed neither mobilization of bone minerals nor hypertrophy of the Stannius corpuscles.

CARBOHYDRATE METABOLISM

Eels starved up to 30 weeks were able to maintain normal blood glucose and lactate levels. Blood glucose was not affected by gonadal development but lactate increased in both sexes as the GSI >20% (Figure 3). Blood pyruvate decreased only in the male eel as the testis developed. The glycolytic key enzymes, hexokinase and pyruvate kinase, were well maintained in liver and white muscles during 30 weeks of starvation. Liver hexokinase was elevated in both sexes as the gonads >10%, while liver pyruvate kinase increased in female eel only. Muscle pyruvate kinase decreased with starvation but was maintained in treated eels. Muscle lactate dehydrogenase tended to increase as starvation progressed but showed no further changes as the gonads developed.

The glycogen concentration in the ovary increased as the oocytes began exogenous vitellogenesis. At a GSI of 30%, the total ovarian glycogen was

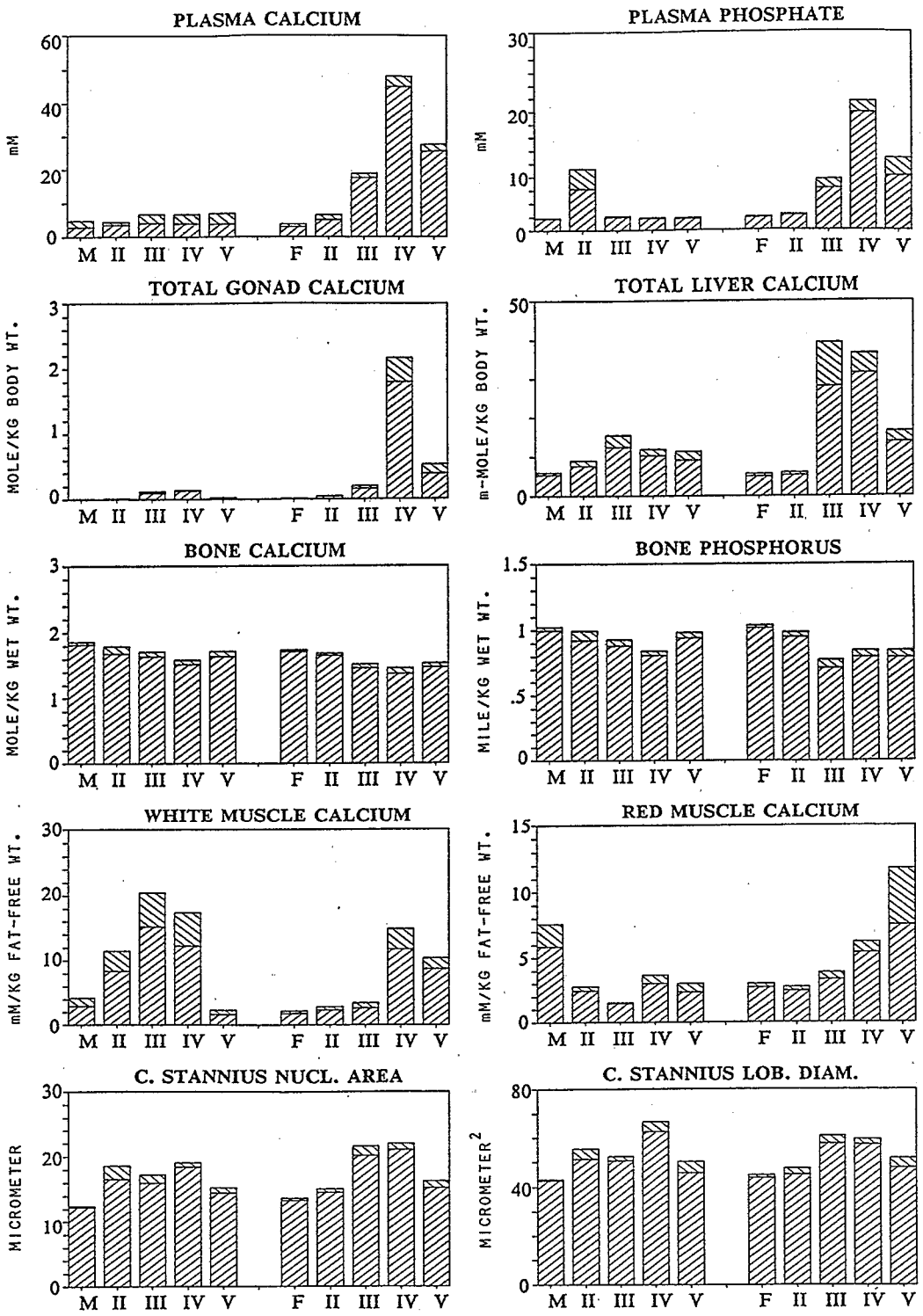

Figure 2. Changes in plasma, gonad, liver, muscle and bone calcium and phosphorus and the corpscle of Stannius in male and female eels during induced gonadal development (M=male control, F=female control; II, III, IV, V=gonadal stage) (mean+SEM).

over 40-fold that of the control ovary. Liver and muscle glycogen store was maintained with starvation but became depleted in the female eel as exogenous vitellogenetic activity began in the ovary. As the animals were starved, the gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-bis-phosphatase were progressively elevated, but gonadal development had no further effect on these enzymes (not shown in figure).

LIPID METABOLISM

The ovary accumulated lipids during the endogenous and the initial phase of exogenous vitellogenesis, reaching peak values as the GSI reached 10% (Figure 4). At this time, the amount of lipid in the ovary was at least 60 times that in the testis of the same weight. Thereafter, the ovaria lipid concentration declined and the total fat in the ovary levelled off.

Free fatty acid levels in the plasma were elevated as the animals were starved. Female control eels had much higher lipid content in the liver than did males. Starvation caused a decline in liver total lipid towards levels found in the male by 20 weeks, but muscle fat content was unaffected by starvation in both sexes. In the male, the liver lipid content rose markedly in all groups treated with HCG reaching peak values at GSI of 10%, which was 4 to 5 fold that of controls. In the female, the liver underwent fatty infiltration during endogenous vitellogenesis and early exogenous vitellogenesis. During this period of stimulated hepatic lipogenesis, the pentose-shunt enzymes (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), malic enzyme and cytosol NADP-isocitrate dehydrogenase in the liver increased significantly in both sexes. As the gonads increased beyond 15% body weight, the amount of lipid in the liver and white muscle began to decline, especially in the female eel; lipid in the red muscle was unaffected compared with that observed in the starved controls.

PROTEIN AND PURINE METABOLISM

Plasma protein increased significantly in female eels as exogenous vitellogenesis was initiated due to the vitellogenin released from the liver (Figure 5). Liver protein levels calculated on a fat-free wet weight were unaltered in the male eel but increased in the female. White muscle protein content declined significantly as GSI >10%, especially in male eels. Both white and red muscle became hydrated. Eels that showed little gonadal response to the hormonal treatment still showed accelerated loss of white muscle proteins. Plasma-free amino acids increased progressively with starvation. In male eels, the levels increased further as the testis enlarged and was 3 to 4-fold higher than the control throughout testis enlargement. In female eels, the levels were unaltered.

Glutamate dehydrogenase was reduced markedly in the liver as gonads developed in both sexes but rose in white muscle of male eels only. AMP aminohydrase of white muscle was markedly elevated in both sexes. The hepatic and muscle uricolytic pathway enzymes such as uricase, allantoinase, allantoicase (only uricase was shown, others had similar patterns) were markedly elevated only in male eels.

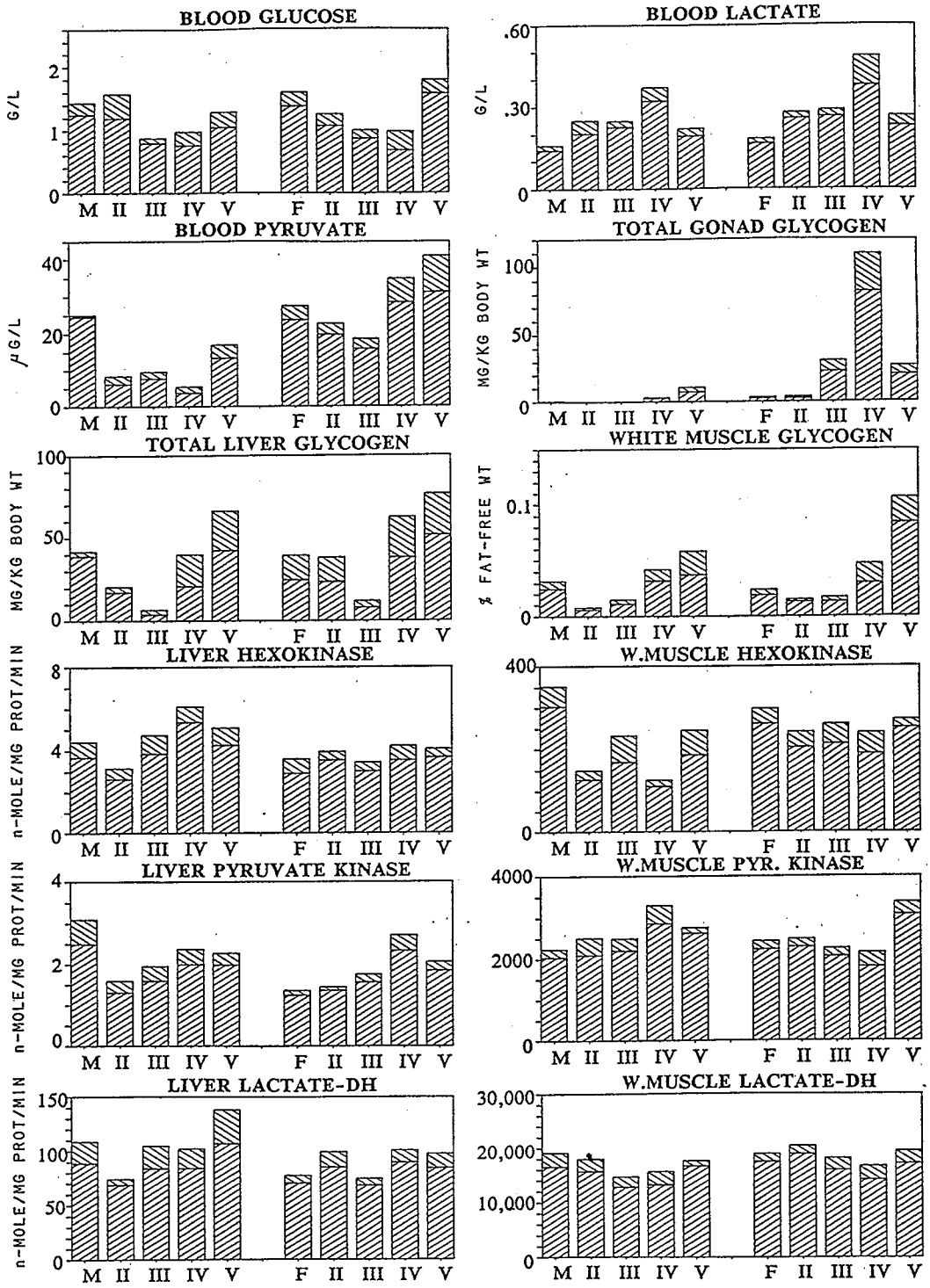

Figure 3. Changes in carbohydrate metabolism in male and female eels during gonadal development: blood, glucose, lactate and pyruvate; gonad, liver and white muscle glycogen content; liver and white muscle hexokinase, pyruvate kinase and lactate dehydrogenase activities (M=male control, F=female control; II, III, IV, V=gonadal stages) (mean+SEM).

DISCUSSION

GONADAL RESPONSE

Like other eel species (Boëtius and Boëtius 1967), the testis of the Japanese eel responded well to HCG treatment. In *Anguilla anguillla*, female eels typically reached a GSI of about 5 to 8% body weight after prolonged treatment with HCG (Boëtius et al. 1962) or purified salmon gonadotropin (Dufour et al. 1984). The failure of ovarian development beyond the iniital stages of exogenous vitellogenesis was due to the lack of gonadotropin release from the pituitary GTH cells. Administration of the releasing hormone (GnRH) or the inhibiting hormone (GnIF) inhibitor such as pimozide stimulated gondotropin release and ovarian development (Dufour et al. 1987). but the GSI still reached only 6 to 8% body weight. Failure to respond to injected gonadotropin has been attributed to the puberty phenomenon with the GnRH-GTH axis being refractory to stimulation when the eel was too small or too young. Carp pituitary extract was much more effective in inducing ovarian development, however, and GSI >30% was obtained in this species after several months (Fontaine 1964a).

Using Japanese eels caught from the same source as the present study, Zhang (1988) conducted a series of experiments comparing the effects of carp pituitary extracts, HCG, LHRH-A, and GnIF-blockers (dopamine antagonists) on gonadal development. Results comparable to those reported in this study were obtained by a combined injection of carp pituitary/HCG/LHRH-A (GSI of45% in 30 weeks), although carp pituitary alone would bring about substantial response over the same period (GSI of 32%). LHRH-A or pimozide/domperidone givenalone was not very effective. Oestradiol-17ß alone or in combination with LHRH-A increased pituitary and serum GTH levels and serum vitellogenin levels, but uptake of yolk protein in the oocyte did not occur and GSI remained low. Differentiation of gonadotrophs during induced gonadal development has been shown previously (Yamamoto and Naghama 1973). Both oestrogen and LHRH stimulate the pituitary GTH cells showing that the positive feedback system was operative in the eel (Olivereau et al. 1986) but is inadequate to bring about full oocyte development. The present study showed that stimulation of gonadotrophs in the pituitary was a prerequisite for the gonadal response to exogenous gonadotropins. Other pituitary hormones present in the carp pituitary or produced endogenously (e.g. TSH) must be important also. Exophthalmia, melanogenesis and thicking of the skin seen in maturing eels are all well established indexes of increased thyroid function in the eel (Etienne 1959).

CORPUSCLES OF STANNIUS, BONE DEMINERALIZATION AND Ca/P BALANCE

Olivereau (1961) first reported stimulation of the corpuscles of Stannius following the injection of carp pituitary extracts or TSH. Fontaine (1964b) showed that surgical corpuscular removal (stanniectomy) caused a marked rise in plasma Ca and K levels and a decline in Na levels. Because of the osmoregulatory effects, much attention had been paid to the role of this gland in calcium and iono-regulation. In the anadromous eel, the immature

yellow eel had small corpuscles, but the migrating silver eels had bigger corpuscles showing signs of stimulation (Hanke et al. 1969). This was interpreted as preparation in anticipation for the ionic challenge when the fish entered seawater. In the catadromous salmonids, stimulation of the corpuscles also was noted at the beginning of the spawning migration and degenerative changes were recorded after spawning (Heyl 1970). Again, these observations were attributed to a decrease in ionic challenge in freshwater (Carpenter and Heyl 1974). Plasma calcium, however, rose in both seawater and freshwater stanniectomized eels but failed to do so in eels maintained in distilled water (Chan et al. 1967). The hypercalcemia was transient, returning to normal within several weeks, but plasma phosphate decreased (Fontaine 1967; chan 1968, 1972).

Stanniectomy did not raise dialysable plasma Ca levels in the European eel and Japanese eel (Chan and Chester Jones 1978, Chan 1972) and a decline in urinary Ca excretion was found in these species (Chan et al. 1969, 1972). In contrast, plasma dialysable Ca increased in the American eel in Ontario and urinary Ca increased, which could be reduced by keeping eels in distilled water (Butler 1969, Fenwick 1974a). This apparent discrepancy probably arose from the difference in Ca level in the environment because Sheffield and Hong Kong tapwater is soft (Ca concentration about 100 uM) but Ontario tapwater is hard. The killfish *Fundulus heteroclitus* was much more sensitive to ambient Ca levels, which directly affected the hypercalcemic response to stanniectomy (Pang et al. 1974). Stanniectomy indeed increased Ca influx through the gills, and the corpuscular hypocalcemic factor (hypocalcin or teleocalcin) inhibited calcium uptake. This was found to operate in salmonids as well as in eels (Fontaine et al. 1972, Fenwick and So 1974).

The inhibition of Ca fluxes and branchial Ca^{2+}-AtPase has been used extensively as a bioassay for the hormone (Copp et al. 1985). Cultured corpuscular cells responded to manipulations of the Ca level in culture medium, discharging secretory products in high Ca media (Aida et al. 1980). The evidence for the Ca-regulating role for the Stannius corpuscles hormone is thus overwhelming. Teleost fish require large amounts of Ca during somatic growth because of the presence of Ca and phosphate mineral deposits in bone and scales, and the Stannius corpuscles can function as a regulator of Ca uptake from the environment, shutting down excessive Ca influx to adjust to the internal demand. Over the short term, intravenous injection of Stannius corpuscles extracts could reduce plasma Ca without affecting plasma inorganic phosphate concentrations (Kenyon et al. 1980).

The present study, however, demonstrated that large amounts of Ca were needed for ovarian development, and plasma Ca levels remained high throughout exogenous vitellogenesis but not so for testicular development. Thus, if the primary physiological role of the Stannius hypocalcemic factor is to suppress plasma Ca leves, one should expect different responses in the two sexes. Furthermore, the inhibition of branchial Ca uptake by the Stannius hormone would limit the source of Ca from the water for the female eel. Yet the corpuscles of Stannius were extremely active (hypertrophy, hyperplasia and degranulation) in both sexes during induced gonadal development. Our previous work on induced gonadal development in eels maintained in freshwater demon-

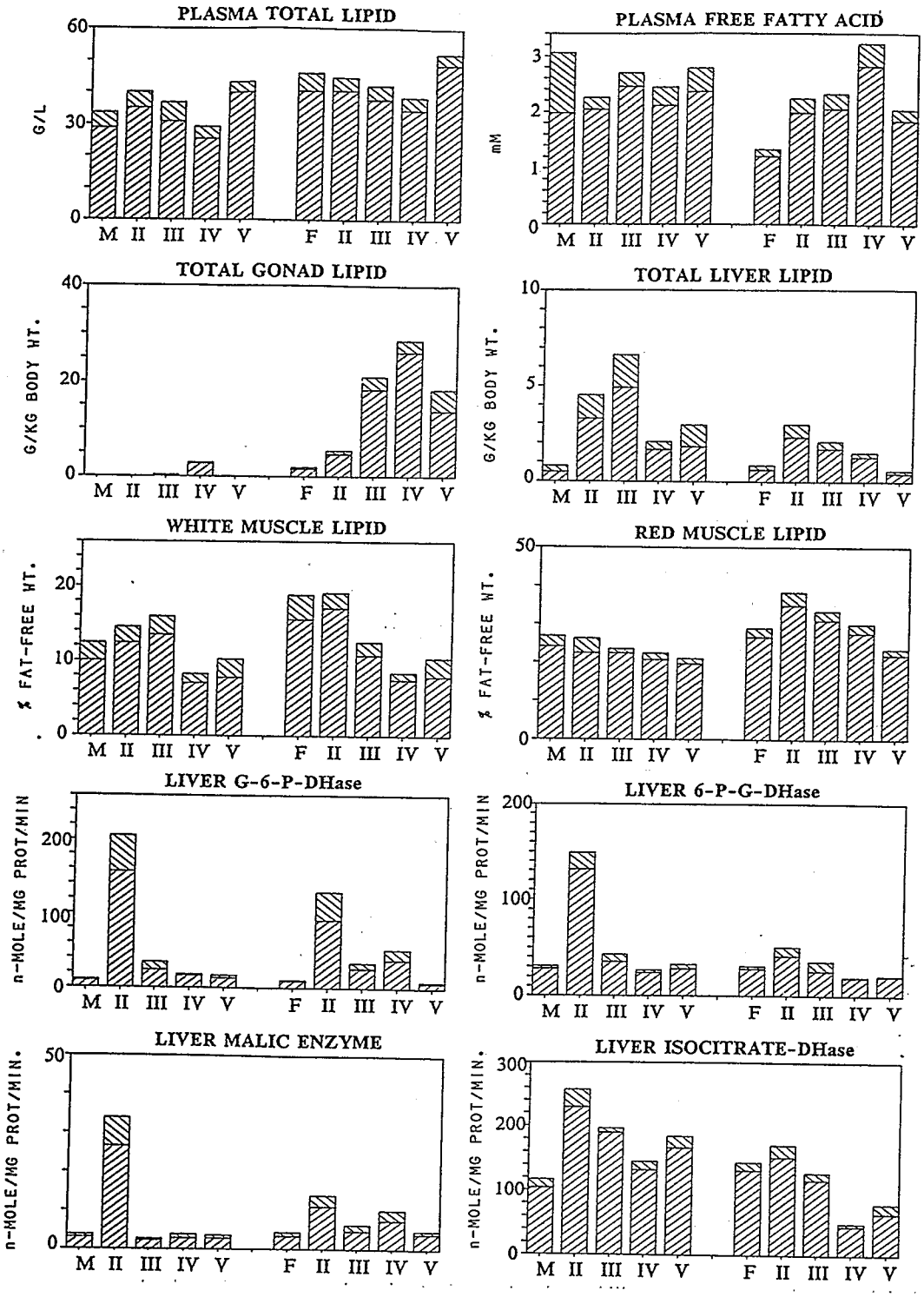

Figure 4. Changes in lipid metabolism in male and female eels during induced gonadal development; plasma total lipid, free fatty acid levels; gonad, liver, white and red muscle lipid content; and liver NADPH-generating enzyme activities (M= male control, F=female control; II, III, IV, V=gonalal stage) (mean+SEM).

strated that the Stannius corpuscle hypertrophic response was even more active in freshwater where the environental Ca was low (Subhedar et al. 1981).

From the present study, it was beyond doubt that the amount of Ca available for the gonads was actually in excess, especially in the male where deposition of Ca in the muscles began early in testis development, whereas in the female eel this occurred when GSI >20%. The picture is entirely different if we consider the supply of phosphorus, which is required in enormous amounts for nucleic acid and phospholipid synthesis (especially in the testis) and vitellogenesis. As the animal is starved, phosphorus is in extremely short supply. The present data clearly demonstrated that this large source of P (along with Ca) was endogenous and derived from demineralization of the bone, which contained at least 92% of the total Ca and 75% of the phosphorus in the body (Fenwick 1974b). Starved eels have been shown to decrease in length suggesting mobilization of resources from bone. Our own unpublished data show that deposition of Ca in soft tissues was pronounced in eels starved for over one and half years. Like the maturing eels, the eel exposed to long-term starvation must resort to bone demineralizttion for its phosphorus supply. Again, the corpuscles of Stannius hypertrophied in these animals. All these data strongly suggest that the primary physiological role of the corpuscles of Stannius is to regulate the phosphate supply from body reserves.

A possible role of the corpuscles of Stannius on bone remodelling has been suggested by Lopez (1970) who showed that Stanniectomy cause the disappearance of osteoclastic activity and loss of amorphous bone in the eel (Lopez 1970b). Using ^{45}Ca labels, Lwowski (1978) suggested that hypercalcemia resulting from stanniectomy probably arose from reduction in incorporation of Ca into bone while release of ^{45}Ca from pre-labelled old bone was unaffected. Bone mineral is deposited mainly as calcium phosphate complexes and part of the deposit is in equilibrium with the plasma Ca and phosphate levels (Fenwick 1974b). Newly formed bone mineral is amorphous and in the octacalcium phosphate form ($Ca_8(PO_4)_3$) with a Ca/P ratio of 1.33 (Hirshman and Sobel 1965), but as the crytal grows $CaCO_3$, other stoichiometric forms of calcium phosphate are added and the Ca/P increases.

In the present study, control eel vertebrae contained a Ca/P of 1.8, which became elevated to 2.0 as demineralization progressed. This pattern is in line with the disappearance of amorphous bone from the mineral complex. Corpuscles of Stannius from immature eels contained only one type of cells containing dense cytoplasmic granules (Fujita and Honma 1967). Based on storage-granule staining and ultrastructure, two types of cells have been described in the Stannius corpuscles of other teleosts (Nadkarni and Gorbman 1966, Krishnamurthy and Bern 1974, Wendalaar Bonga et al. 1975). More recent immunochemical study using anti-hypocalcin showed, however, that these represented two stages of the same cell type (Kaneko et al. 1988). The present study supports the notion that only one cell type is present in the Stannius corpuscles and that the enlarged cells devoid of dense storage granules represent the active secretory state.

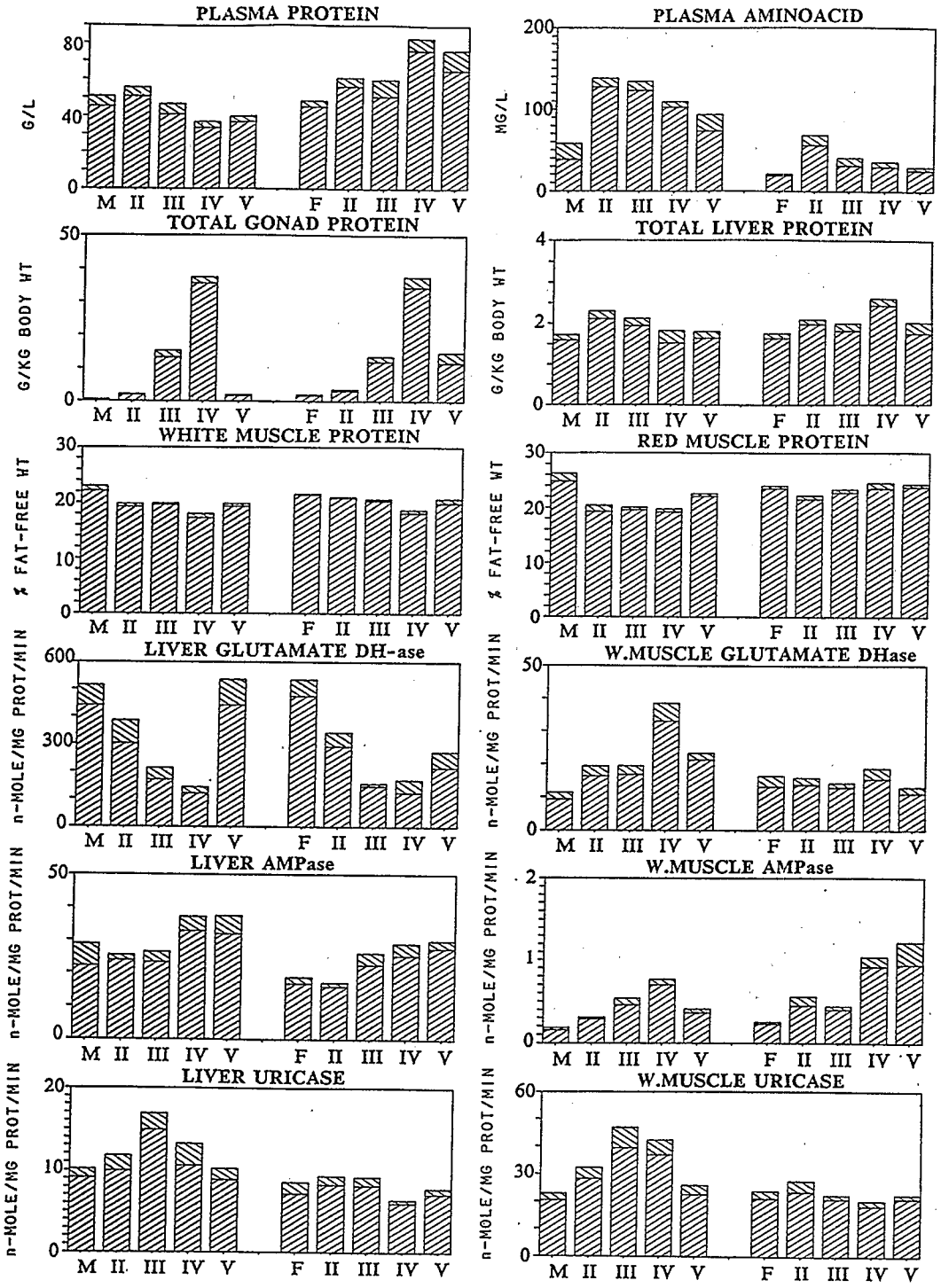

Figure 5. Changes in protein and purine metabolism in male and female eels during induced gonadal development: plasma protein and free aminoacid levels; gonad, liver and muscle protein content; liver and white muscle glutamate dehydrogenase, AMP aminohydrase and uricase activities (M=male control, F=female control; II, III, IV, V=gonadal stages) (mean+SEM).

MOBILIZATION OF BODY RESERVES

Although data on changes in body weight and length and in body compartment sizes (liver, gut) have been recorded for eels undergoing induced gonadal maturation, not much is known about changes in biochemical composition and the enzymes involved in mobilization of resources. The migrating silver eels certainly differed in biochemical composition from the yellow eel, especially in their higher fat content, and these differences have been attributed to natural gonadal development (Lewander et al. 1976). Starved eels lost between 0.1 and 0.2% body weight per day (Inui and Oshima 1966; Chan et al. 1969, 1980) with about 30% of total energy derived from protein catabolism and 70% from oxidation of fat (Fisher 1977, Chan and Woo 1978). The bulk of the body reserves in support of metabolism came from mobilization of protein and fat from white muscles (Inui and Oshima 1966, Aster and Moon 1981). Starved eels have been shown to survive at least 3 years, by which time about 70% of the initial weight had been lost (Boëtius and Boëtius 1967).

With increased mobiliaation of tissue protein and lipid, plasma aminoacid and free fatty acid levels increased as expected. Loss of protein from white muscle during starvation has been shown to originate mainly from the insoluble fiber component (Moon 1983a,b). The soluble protein fraction in liver and muscle that contained the metabolic enzymes was unaltered. Gluconeogenic enzymes (glucose-6-phosphatase, fructose-1,6-bis-phosphatase and PEP carboxykinase) and aminoacid metabolizing enzymes (glutamate-pyruvate transaminase, glutamate-oxaloacetate transaminase and leucine transaminase) in liver tend to be conserved while muscle FDPase and GPT increased (Moon 1983a).

Based on the NADPH-generating capacity, the liver appeared to be the primary site of lipogenesis in the eel (Aster and Moon 1981). In short term starvation (1-3 weeks), there was marked reduction in hepatic fatty acid synthetase and acetyl-CoA carboxylase, but the NADPH-generating enzymes were maintained (Abraham et al. 1982). With prolonged starvation (26 weeks), however,hepatic glucose-6-phosphate dehydrogenase was reduced while most other enzymes were conserved (Aster and Moon 1981). The rate of conversion of ^{14}C-acetate to fatty acid progressively decreaed with starvation up to 39 weeks but significant levels of activity still remained up to at least 95 weeks of starvation (Abraham et al. 1982). Muscle lipid content actually increased steadily during prolonged starvation (Moon 1983a).

Blood glucose and carbohydrate supply was maintained (Dave et al. 1975, Renaud and Moon 1980), but enzymes of the glycolytic pathway and the tricarboxylic acid cycle in the muscles decreased slightly (Boström and Johansson 1972.). Thus in a broad sense, starvation in eels evoked changes that tended to preserve the metabolic enzyme machinery, whereas in other fish species, drastic decreases in liver and muscle enzyme activities would result. The present data showed that the unique enzymatic adaptation to starvation in the eel is put to important use during gonadal development, which takes place without dietary nutrient supply.

The nitrogen requirements of the developing testis differ substantially from that of the ovary, the former being rich in nucleic acids and the latter in yolk proteins. Differences in plasma protein levels in male and female

eels during gonadal development reflected the hepatic synthesis and release of vitellogenin in the female and its absence in the male (Hara et al. 1980). Vitellogenin is a phophoprotein that specifically binds Ca. In male eels, the plasma-free aminoacid levels increased drastically but did not rise in the female eel. This probably represented aminoacid in transit, much of which was removed by the liver for vitellogenin synthesis in the developing female eel. Stimulation of glutamate dehydrogenase and AMP aminohydrolase in white muscle during gonadal development was in line with the elevated breakdown of muscle tissue. Suppression of glutamate dehydrogenase in the liver was probably due to the need to conserve nitrogen. In female eels treated with oestradiol-17ß, mobilization of protein without concomittant gonadal development was accompanied by marked elevation of ammonia excretion and ammonia loss/oxygen consumption ratio in eels maintained in freshwater (Chan and Cheung 1982). For purine catabolism, the enzymes of the uricolytic pathway was elevated only in male eels suggesting marked increase in purine turnover. This probably was related to the marked elevation of nucleic acid synthesis in the developing testis.

Substantial increases in the lipogenic enzymes (NADPH-generating systems) were recorded in both sexes and this was probably due to the large demand on lipids for gonadal development. Increased hepatic lipogenesis occurred early in gonadal development in both sexes, but the large demand of lipid in the developing oocytes during exogenous vitellogenesis later depleted this store in the female eel. The ultimate source of the lipid was certainly the white muscle compartment, which showed a marked decline in fat-store in female eels. Unlike other teleost fish, the eel has little abdominal fat and red muscle fat content was unaffected during gonadal development.

The present study showed that the key enzymes in intermediary metabolism were unimpaired and in some cases elevated during induced gonadal development. In contrast, the marked decline in haematocrit values placed the animal in jeopardy and probably constituted the most important factor limiting survival.

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UTILIZATION OF INORGANIC NITROGEN BY TILAPIA NILOTICA

by
Wang Yi Qiang[1] and Shu Xue Bao[1]

INTRODUCTION

The development of animal culture throughout the world requires large sources of protein. Whether the protein source can be supplied sufficiently will affect this development directly. Thus to exploit protein sources is an outstanding problem.

The use of NPN by organisms to synthesize protein was examined in ruminants in the late 19th Century. Urea and other nitrogen compounds can be utilized by ruminants. These compounds may be decomposed into ammonia by microorganisms in the rumen of the animal; ammonia, then, becomes an effective protein source through synthesis by microorganisms. So it is considered that urea can be used to replace a part of protein in the diet.

Urea-contained nitrogen is about 2.8 times that of protein-contained nitrogen, and the price of urea is low. So several hundred thousand tons of urea are used each year in animal feed in the USA. Molimoto (1971) reported that urea might be used to replace about 20 to 30 percent of protein, resulting in greater economic efficiency.

Urea was used by microorganisms to synthesize protein, which was supplied to the animal. The urea was not utilized by the animal directly.

Animals have different dietary natures requiring different levels of dietary protein. Some animals that require little protein grow rapidly. Obviously, a part of protein is synthesized in the animal body. It may be that the non-protein substance in the animal body, under the condition of existence of nitrogen, transforms into protein. Wang Yi-Qiang et al. (1987), using an immunocytochemical method, confirmed that the grass carp (Ctenopharyngodon idellus) and the black carp (Mylopharyngodon piceus) have different levels of insulin in their islets of Langerhans. Evidently, their levels of metabolism of carbohydrates are different. Grass carp use more carbohydrates to transform into protein and, therefore, they should be provided with more nitrogen to increase growth. So, whether the animals can or cannot use NPN directly to synthesize into protein is a problem for discussion.

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Fishes have different dietary natures and require different protein levels in diet also. Satia (1974) proved that rainbow trout require 40 to 46%. Ogino and Saito (1970) and Takeuchi et al. (1979) discovered common carp require 31 to 38%. Lin Ding et al. (1980) and Yang Guo-Hua et al. (1985) reported grass carp need 20 to 25%. Garling and Wilson (1976) proved catfish need 22 to 40%. Jauncey (1982) confirmed that tilapia require 35%. There are contrary opinions among scientists concerning whether fish can use NPN directly. Nilni and Keveren used a diet containing urea to feed carp and catfish. They proved that fish cannot utilize inorganic N. Fladofska and Valet used urea to replace the protein source to feed the carp and mullet. They proved that urea can replace a part of protein in the diet. Chen et al. (1986) made a special diet containing urea to feed carp. They got a high output.

All the different scientific opinions were obtained from studies of growth by adding NPN to the diet. The investigators examined whether the fishes can utilize NPN directly to replace the protein in diet. None of them investigated the physiological activity of the animal.

This paper studied the amount of NPN directly through the digestive tract or peritoneal cavity of the fish. The utilization ratio of absorbed NPN in fish was measured by means of stable nuclide ^{15}N-labeled NPN. The results will provide scientific basis for fisheries production.

MATERIALS AND METHODS

Tilapia nilotica (spawned juvenile) were taken from the fish farm of our university and were transferred to a circulating, filtrating aquarium for provisional rearing. The size of aquarium is 200 x 80 x 80 cm^3. The length of fishes was from 5 to 13 cm; the weight was from 3 to 33 grams.

The pellet diet was made containing the high protein level and normal protein level by the materials according to the formula as Table 1.

TABLE 1. THE DIET FORMULA (percent)

	Fish meal	Bran	Rice chaff	Flour	Plant oil	Mineral	Rape seed cake	Vitamin compound	Protein
High protein level diet	53	8	5	4	3	4	23	0.01	49.87
Normal protein level diet	15	30	20	9	3	4	19	0.01	27.62

$^{15}NH_4Cl$ (made by Shanghai Chemical Industrial Institute), abundance 95.44%, was added to the diet.

The fishes were fed a pellet diet containing 30% protein until they ate it at once when the food was put in the water. After acclimation to the diet, the fish were divided into three groups: a high protein level diet group, a

normal protein level diet group, and a starved group. Before the experiment, the diet of both the high protein level diet group and normal protein level diet group, which did not receive $^{15}NH_4Cl$, were used for 15 days. The starved group also was fed with normal protein level diet for 8 days, then starved for 7 days. Then the test began.

The fish were divided into a feeding group and an injection group for introducing $^{15}NH_4Cl$ into the organisms. In the feeding group, the fish were fed a high protein level diet and a normal protein level diet, both of which contained $^{15}NH_4Cl$. The amount was 0.5 g/fish/day. From the first time of feeding, three fish were taken for sampling from each group.

In the injection group, a 30% $^{15}NH_4Cl$ injection solution was introduced at a rate of 0.3 mg NH_4Cl per gram of body weight through intraperitoneal cavity injection. After injection, three fish were taken from each group for analysis. During the whole experimental time, the fish were fed either a high protein level or a normal protein level diet not containing $^{15}NH_4Cl$.

The temperature of the water was 24 to 25°C; the dissolved oxygen content was 3.5 to 5.5 mg/L.

Fish removed for sampling were dissected at once and all muscle weighed. The amount of N in a certain weight of muscle was determined by Kjeldahl's method. Another portion of the muscle was treated twice with 5% trichloroacetic acid to precipitate the protein and then N was determined using Kjeldahl's method. The abundance of ^{15}N in each sample was determined by mass spectrometry.

Formulas used in the calculation were: $\Delta N_1/N_1 = \frac{R_G - R_i}{R_f - R_i}$

$$N\% = \frac{\Delta N/N - \Sigma N_1}{\Sigma N_2}$$

where: $\Delta N/N$ = the amount of NH_4Cl-N absorbed ($\Delta N_1/N_1$) or transformed into protein ($\Delta N_2/N_2$) within each gram protein-N of fish muscle

R_G = abundance of ^{15}N in muscle sample

R_f = abundance of $^{15}NH_4Cl$ in diet or injected solution

R_i = the natural abundance of ^{15}N in fish muscle

ΣN_1 = the total amount of N in fish muscle

ΣN_2 = the amount of NPN (mg) induced by eating or injection

RESULTS AND DISCUSSION

Tilapia can absorb NH_4Cl-N through the digestive tract, as shown in Figure 1.

In the feeding groups, all the fishes in the high protein level diet group, the normal protein level diet group, and the starved group, showed an increase of N in muscle according to the continuous experimental time. The $\Delta N/N_1$ rose from 0.052, 0.054, and 0.036 mg N/g N at 5 hours to 0.568, 0.380, and 0.518 mg N/g N at 48 hours, respectively.

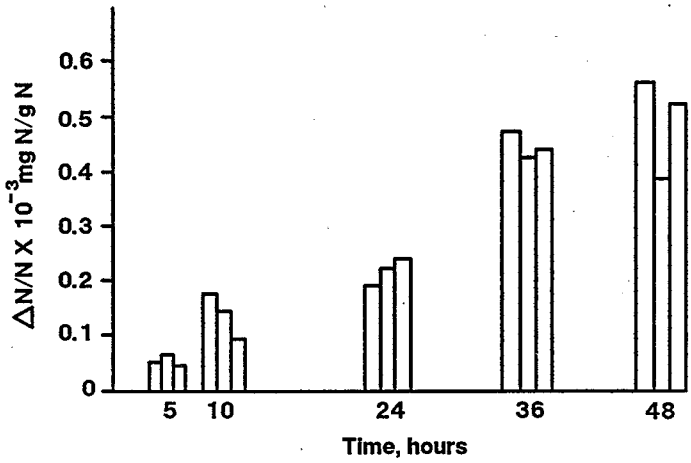

Figure 1. The absorption of NH_4Cl-N through digestive tract at different times.

Tilapia can absorb the NH_4Cl-N through the peritoneal cavity, as shown in Figure 2.

Five hours after injection, the $\Delta N_1/N_1$ was 0.132, 0.224, and 0.155 mg N/g N for the high protein level group, the normal protein level group, and the starved group, respectively. This means that, in tilapia, NPN was absorbed through peritoneal cavity very quickly. From 5 hours to 10 hours, the $\Delta N_1/N_1$ decreased, meaning that, at this time, a certain amount of NPN was excreted. From 10 hours to 48 hours, the $\Delta N_1/N_1$ continued to decrease slowly, which means that NPN may take part in synthesizing into protein.

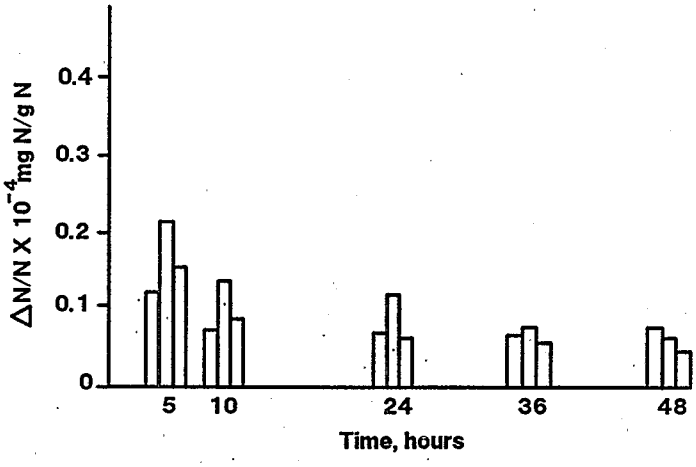

Figure 2. The absorption of NH_4Cl-N through peritoneal cavity at different times.

After the NH_4-N was absorbed, does the N take part in synthesizing into protein? We used trichloroacetic acid to treat the muscle of fish in feeding group in order to precipitate the protein in muscle. We then determined the abundance of ^{15}N and observed its variation and calculated the amount of NPN transferring into the muscle. The result shows that the abundance of ^{15}N in muscle increased according to digestion and absorption (Figure 3).

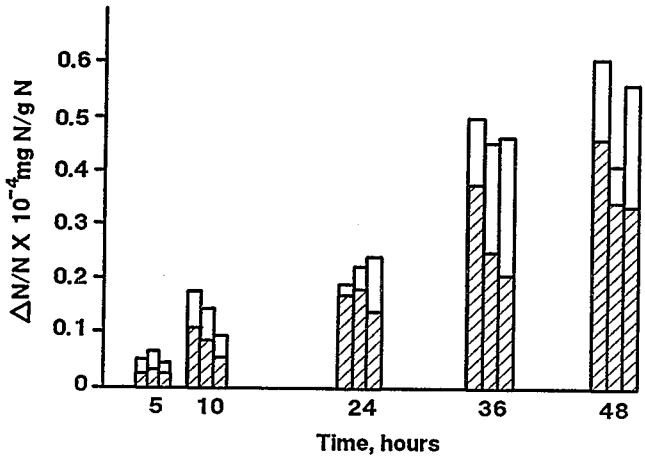

Figure 3. The amount of NPN transferring into the protein of fish muscle and the total amount of absorbed NPN.

In comparing the amount of NPN transferring into the protein of fish muscle ($\Delta N_2/N_2$) with the total amount of absorbed NPN ($\Delta N_1/N_1$), we see that the ratio (R) has risen. At 10 hours, the R values of the three groups are 54.1%, 56.1%, and 55.5%, respectively. At 48 hours, the R values rise to 75.5%, 74.7%, and 60.3%, respectively. It means that, in tilapia, not only the NH_4-N can be absorbed through digestive tract but also the absorbed NPN can be synthesized continuously into the protein of muscle.

Using the feeding amount within 48 hours, the total amount of muscle protein in each fish, its percentage in the total fish protein, and other data, we established the absorptivity of NH_4Cl in 48 hours by tilapia body and muscle and its transferability of protein from the absorbed NH_4-N (Table 2).

A few papers relate metabolism of fish studied with stable nuclide ^{15}N. Yitou studied the juvenile of common carp, crucian carp, loach, etc. absorbing the NH_3-N and NO_2-N from water. He confirmed that the absorbed NH_3-N takes part in metabolism entering various tissue and organs by biosynthesis.

In this paper, we proved that the amount of absorbed NPN in muscle at 48 hours is ten times that at 5 hours; that the absorptivity of NPN in muscle after injection at 5 hours is 4 to 5 times the absorptivity after feeding at 5 hours; and that, by treating with 5 mg NH_4Cl, after treatment at 5 hours, the absorptivity of NPN through the peritoneal cavity is 3 times that through digestive tract. From the R value at 48 hours, we estimate that most of the absorbed NPN took part in the synthesis of muscle protein.

TABLE 2. THE ABSORPTIVITY OF NH_4-N BY FISH BODY AND MUSCLE PROTEIN AND THE TRANSFERABILITY OF PROTEIN FROM NH_4-N IN MUSCLE IN 48 HOURS

	Absorptivity of NH_4Cl, %		Transferability of NH_4-N into fish muscle, %
	Fish body	Fish muscle	
High protein level diet	3.27	1.97	75.5
Normal protein level diet	2.54	1.53	74.7
Starved group	1.77	1.06	60.3

Now it is sure that, in tilapia, the NH_4-N can be absorbed directly and be synthesized into protein. It can be quantified, but the amount of inorganic N replacing protein is very little. In the ruminant, adding 2% urea can replace 20 to 30% protein in the diet. And the dietary coefficient can be decreased to 1.3 by adding 2% urea into the diet of rainbow trout.

Urea is the end excreta of metabolism in mammals. After they are decomposed they can be utilized to synthesize protein by microorganisms. This is a kind of indirect utilization but is not direct utilization as this experiment.

CONCLUSION

In tilapia, the direct absorption of NH_4-N can be both through its digestive tract and the peritoneal cavity. At 5 hours, the absorptivity through the peritoneal cavity is higher than that through the digestive tract. The absorbed NH_4-N took part the synthesis of body protein. In tilapia, at 48 hours, the transferability is 60.3 to 75.5% in the muscle.

In tilapia, the absorptivity of 2% NH_4Cl in diet is 1.77 to 3.27%.

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OXYGEN, CARBON DIOXIDE AND AMMONIA TRANSFER ACROSS TELEOST FISH GILLS

by

David J. Randall[1]

Fish transfer oxygen, carbon dioxide and ammonia across their gills between water and blood. About equal amounts of oxygen and carbon dioxide are transferred, but in opposite directions, whereas ammonia excretion is only 10 to 30% of oxygen uptake. This, of course, is a reflection of metabolic utilization and production of these compounds. The carbon dioxide excretion: oxygen uptake exchange ratio is usually between 0.7 and 1.0, whereas the ammonia:carbon dioxide exchange ratio is between 0.1 and 0.3 (Randall and Wright 1989). Variations in the exchange ratio outside these levels result from changes in body stores of carbon dioxide and/or ammonia.

Body stores of these two compounds represent about two or three times the excretion rate per hour. Oxygen stores, ignoring that in the swimbladder, are only sufficient to maintain tissue requirements for about 5 minutes. If the swimbladder contains only oxygen then, assuming the fish can utilize all the oxygen present, swimbladder oxygen could maintain tissue oxygen requirements for a few hours (see Randall and Daxboeck, 1984, for review). It is possible that swimbladder oxygen stores supplement oxygen requirements during exposure of fish to hypoxic environments (Smith et al. 1983).

The gills consist of a series of gill arches, supporting rows of filaments with lamellae, forming a sieve-like structure in the path of the water flow. The blood circulation is complex having both lamellar and filamental components. The filament circulation is part of a secondary, low hematocrit circulation that serves superficial structures in the fish. Surface structures in fish obtain oxygen from and excrete carbon dioxide and ammonia directly into the surrounding water (Randall and Daxboeck 1984).

Oxygen transfer across the gills into the blood is to supply internal organs only. The secondary circulation to the skin and gill filaments is not to exchange gases but rather to deliver organic nutrients, remove waste-products, and maintain osmotic balance in the tissues. In resting fish, skin and gill oxygen consumption represents about 20 to 30% of total oxygen uptake

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by the fish; that is, only 70% of oxygen consumed by the fish crosses the gills into the blood, the rest is supplied to superficial structures directly from the water. The fraction of total oxygen uptake crossing the gills will increase with exercise because the increase in oxygen uptake goes almost entirely to the working muscles that receive their oxygen via the blood.

Blood flows through the lamellae countercurrent to the water flow. Blood flows around pillar cells, which act as posts, embedded with collagen that extends around the blood space, holding the two walls of the lamellae parallel to each other (Figure 1a). The blood sheet is about 9 to 12 um thick and is very dependent on blood pressure. The lamallae show no increase in length or height as blood pressure rises but the width does increase with blood pressure (Farrell et al. 1980). Gas transfer occurs across the lamellar wall, which consists of a pillar cell wall, a basement membrane, and two layers of epithelial cells.

There are intracellular spaces between epithelial cells but the outer layer of cells are bound together by tight junctions, ensuring a low osmotic and ionic permeability of the respiratory epithelium. Mucus covers the apical surface of the epithelial layer and water flows between the lamellae in a sheet about 25 um thick. Blood transit time through the lamellae is about a second whereas that for water is about 100 milliseconds.

Oxygen uptake across the gills is both perfusion and diffusion limited (Randall 1982). Increases in oxygen uptake are achieved by increases in both blood and water flow and the diffusing capacity of the gills. Maximum oxygen delivery to the tissues is determined by cardiac output and arterial oxygen content (Jones 1971). Many teleost fish have blood with a marked Root shift, that is, a decrease in pH reduces the oxygen capacity of the blood even at high Po_2. The Root shift plays an important role in unloading oxygen into the swimbladder.

Acidotic conditions, therefore, could lead to a reduction in oxygen delivery to the tissues by reducing blood oxygen content. This is ameliorated, however, by the release of catecholamines into the blood. Catecholamines react with beta-receptors on red blood cells and stimulate sodium/proton exchange. This causes a rise in intracellular pH and an increase in intracellular sodium and chloride levels, which results in swelling of the erythrocytes as water is sucked into the cells (Nikinmaa et al. 1987). The elevation in erythrocytic pH, due to stimulation of sodium/proton exchange by catecholamines, offsets the effect of a reduction in plasma pH on erythrocytic pH and contributes to the maintenance of arterial blood oxygen content (Primmett et al. 1986, Randall et al. 1987).

Carbon dioxide is excreted as CO_2, but a small fraction (about 10% of total carbon dioxide excretion in resting fish) is converted to bicarbonate in the gill epithelium and exchanged for chloride across the apical membrane (Figure 2). There appears to be no functional carbonic anhydrase activity in the plasma or associated with the respiratory endothelium (Perry et al. 1982). In trout, and probably other fish, there is no carbon dioxide excretion in the absence of red blood cells. Under conditions of controlled blood flow and carbon dioxide content, excretion is proportional to hematocrit (Perry et al.

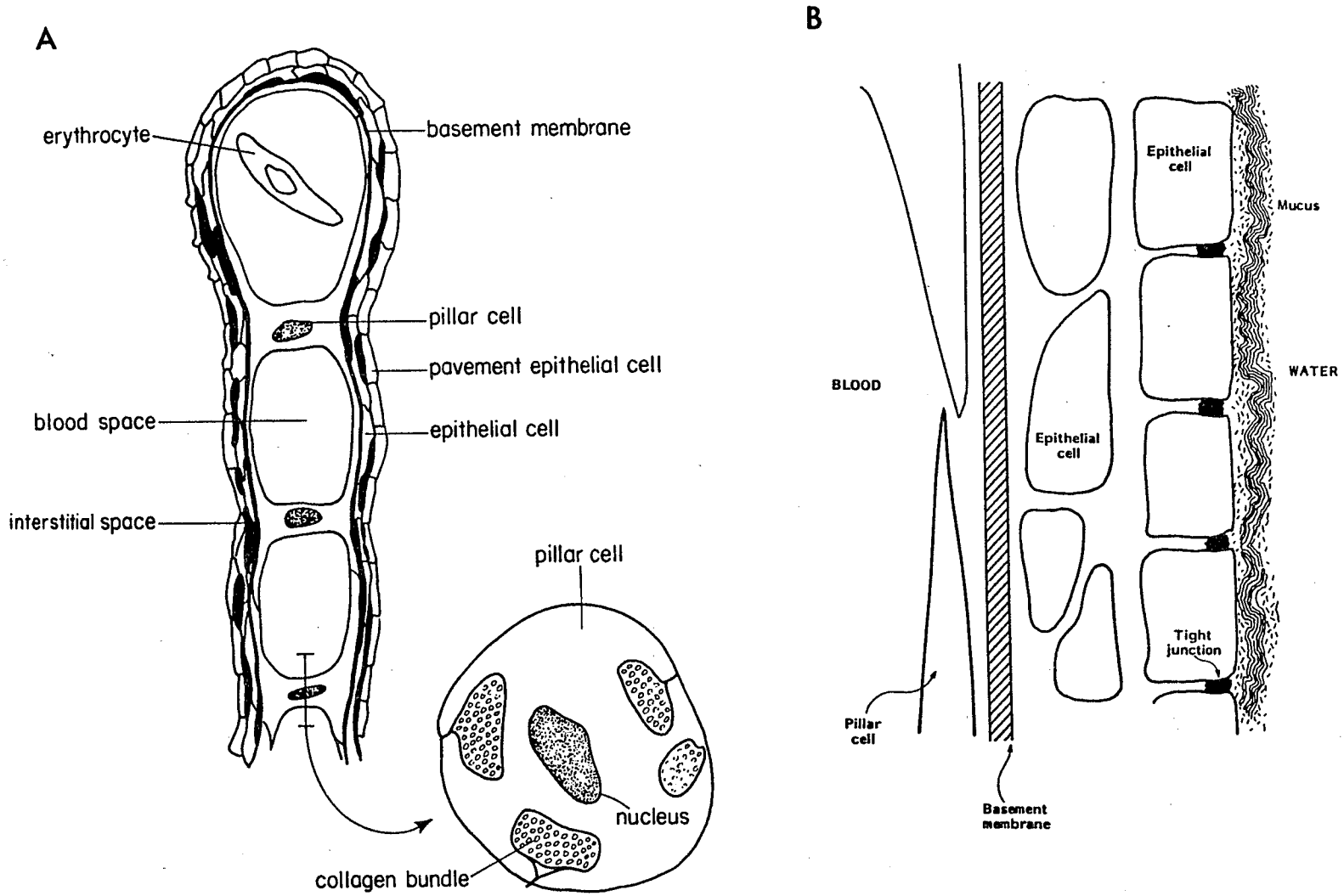

Figure 1. A--cross section through the lamella and a pillar cell of a trout gill (after Randall 1982). B--section through the blood:water barrier of the gill of a fish.

1982). Carbonic anhydrase activity in fish red blood cells is high and is required to catalyse the bicarbonate dehydration reaction in blood in the gills.

The erythrocyte membrane also contains high concentrations of the protein associated with chloride/bicarbonate exchange (Table 1). That is, red blood cells are required to ensure adequate rates of bicarbonate dehydration, during blood transit of the gill, and to maintain low levels of carbon dioxide in the blood. The gill epithelium also contains high levels of carbonic anhydrase, this enzyme appears to function in maintaining adequate fluxes of protons and bicarbonate, through the catalysis of the carbon dioxide hydration reaction, for anionic and cationic exchange processes on the apical surface of the gill (Figure 2).

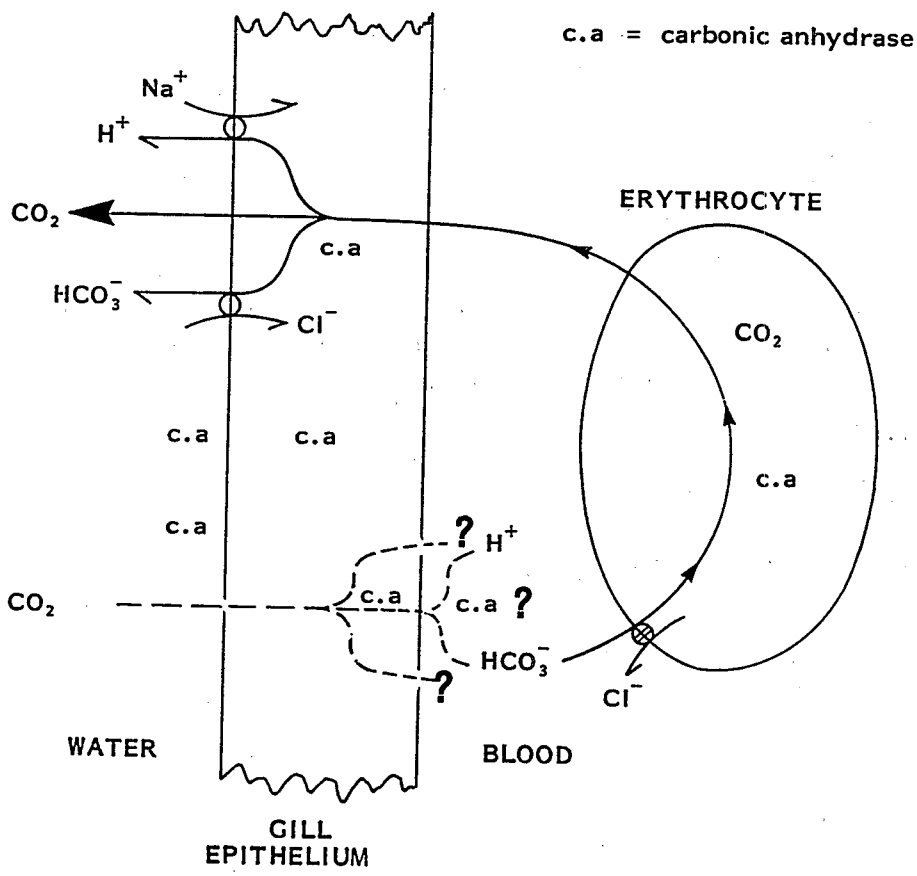

Figure 2. Diagramme to illustrate the pattern of carbon dioxide excretion across the gills of teleost fish. The dotted line represents possible but unlikely pathways of carbon dioxide excretion in fish.

TABLE 1. CHARACTERIZATION OF CHLORIDE/BICARBONATE TRANSPORT SYSTEM (BAND III MOLECULES) IN TROUT RED BLOOD CELLS COMPARED WITH THAT FOR HUMAN RED BLOOD CELLS (from Romana and Passow 1984)

	Trout	Human
Band III molecules, $cell^{-1}$	8×10^6	1×10^6
Cell surface, cm^2	2.67×10^{-6}	1.42×10^{-6}
Band III molecules, cm^{-2}	30×10^{11}	7×10^{11}
Half time for Cl^- ion exchange, s:		
0°C	3.42	17.2
10°C	1.29	2.32
15°C	0.81	0.89
38°C		0.05

Carbon dioxide and ammonia are the major metabolic endproducts excreted across the gills of fish. In general, the fish excretes about ten times as much carbon dioxide as ammonia. These compounds exist in the body in both an ionized and unionized form. The ratio of carbon dioxide (CO_2) to bicarbonate (HCO_3^-) and ammonia (NH_3) to ammonium ion (NH_4^+) varies with pH, but the pKs of these reactions are very different. The pK of the NH_3/NH_4^+ reaction is about 9.5, whereas the apparent pK of the CO_2/HCO_3^- reaction is around 6.1 (Boutilier et al. 1984). The CO_2/HCO_3^- and NH_3/NH_4^+ ratios are equal at the pH where the two lines cross in Figure 3A, at the midpoint between the pK of the NH_3/NH_4^+ reaction and the apparent pK of the CO_2/HCO_3^- reaction. The pH at this point is similar to the pH of fish blood over a range of temperatures. The midpoint between these two pKs varies with temperature in much the same way as blood pH changes in fish. This is of functional significance, for the animal must maintain the transfer of both ammonia and carbon dioxide out of the body. [The term carbon dioxide refers to total carbon dioxide (CO_2 + H_2CO_3 + HCO_3^- + $CO_3^=$), and ammonia to total ammonia (NH_3 + NH_4^+)].

The gill epithelium is not very permeable to either HCO_3^- (Perry et al. 1982) or NH_4^+ (see review by Randall and Wright 1987), but is very permeable to CO_2 and NH_3. Thus, CO_2 and NH_3 will be the predominant forms excreted, as long as adequate blood-to-water CO_2 and NH_3 gradients exist. This will occur for both metabolic endproducts if blood pH is maintained at the midpoint between the pK of the two reactions and environmental levels of NH_3 and CO_2 remain low. The fish must maintain CO_2 and NH_3 excretion because increased CO_2 levels result in an acidosis and because high levels of ammonia in the body are toxic, resulting in convulsions in all vertebrates. A blood acidosis, however, will favour CO_2 excretion whereas a blood alkalosis will augment NH_3 excretion (Hillaby and Randall 1979). Both blood CO_2/HCO_3^- and NH_3/NH_4^+ ratios are very low because blood pH is nearly two orders of magnitude away from the point where pH equals pK for either reaction. Thus in both instances, the unionized form represents only 1 to 5% of the total and there are large stores of HCO_3^- and NH_4^+ in the body.

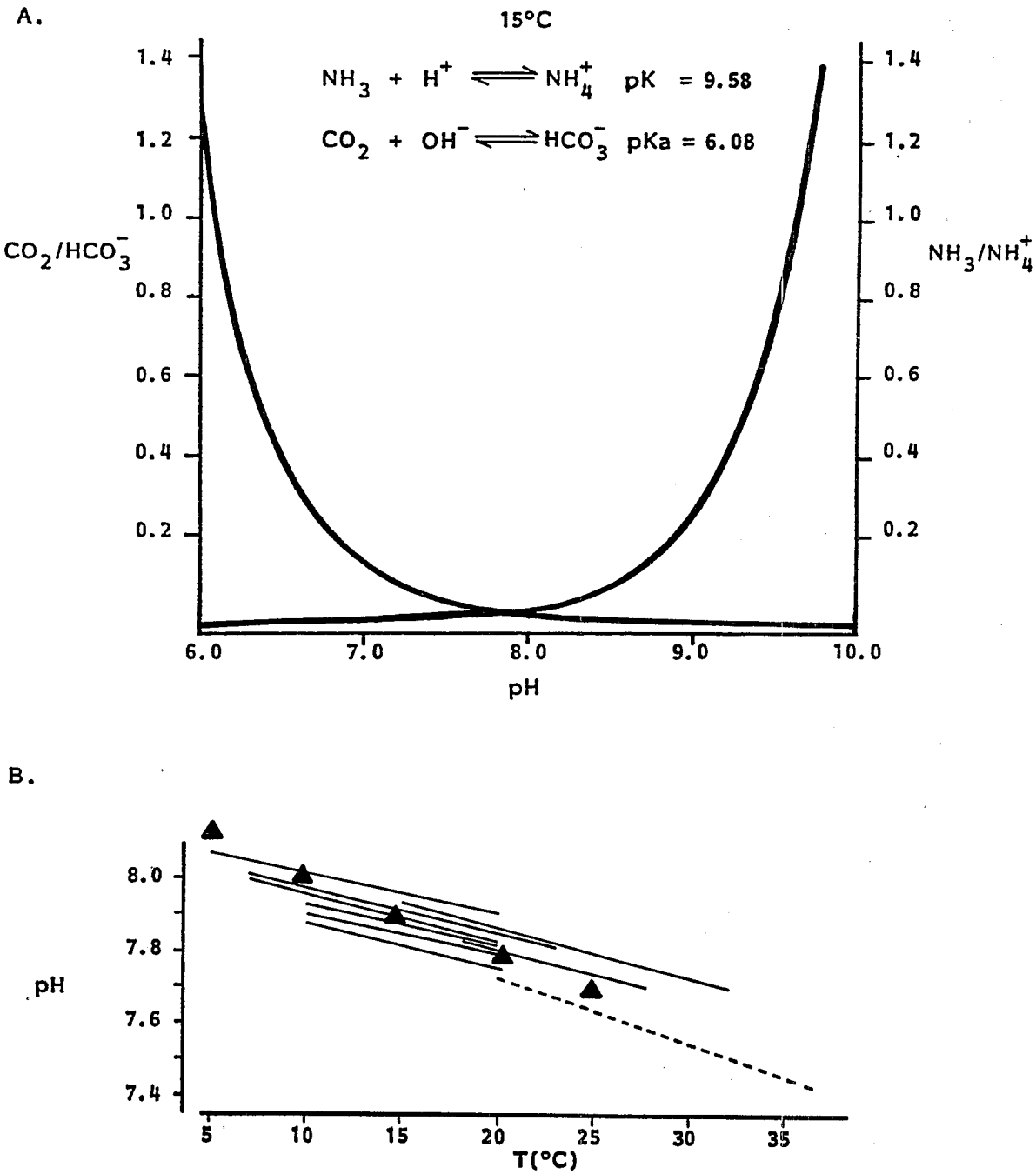

Figure 3. A. The effect of varying pH on the CO_2/HCO_3^- and NH_3/NH_4^+ ratio in trout plasma at 15°C. B. The pH at which the ratios are equal have been calculated at different temperatures and added to a graph of variations in plasma pH with temperature for several fish (from Randall and Wright 1988).

Excretion of CO_2 and NH_3 will be influenced by the composition of water near the gill surface, which may have a different chemical composition from that of the bulk medium. The surface of the gills is covered by a mucous coating and there is also a boundary layer of water next to this mucous layer. Molecular CO_2 and NH_3, along with some HCO_3^- (Perry et al. 1982) and NH_4^+ (Wright and Wood 1985), are excreted into the mucous and boundary water layer next to the gill surface (Figure 4). Bicarbonate ions represent only a small portion of the carbon dioxide excreted, probably less than 10%, and the amount of ammonia excreted as NH_3 varies between 45 and 100% (see review by Randall and Wright 1988). As long as the pH is above 6.1, then most of the CO_2 excreted will form HCO_3^- and acidify the water. Wright et al. (1986) showed that this reaction was catalysed by carbonic anhydrase in the mucous layer.

The mucous layer contains a large number of damaged gill epithelial cells, which are known to contain high levels of carbonic anhydrase (Lacey 1983). CO_2 entering this boundary layer will acidify the mucous and boundary

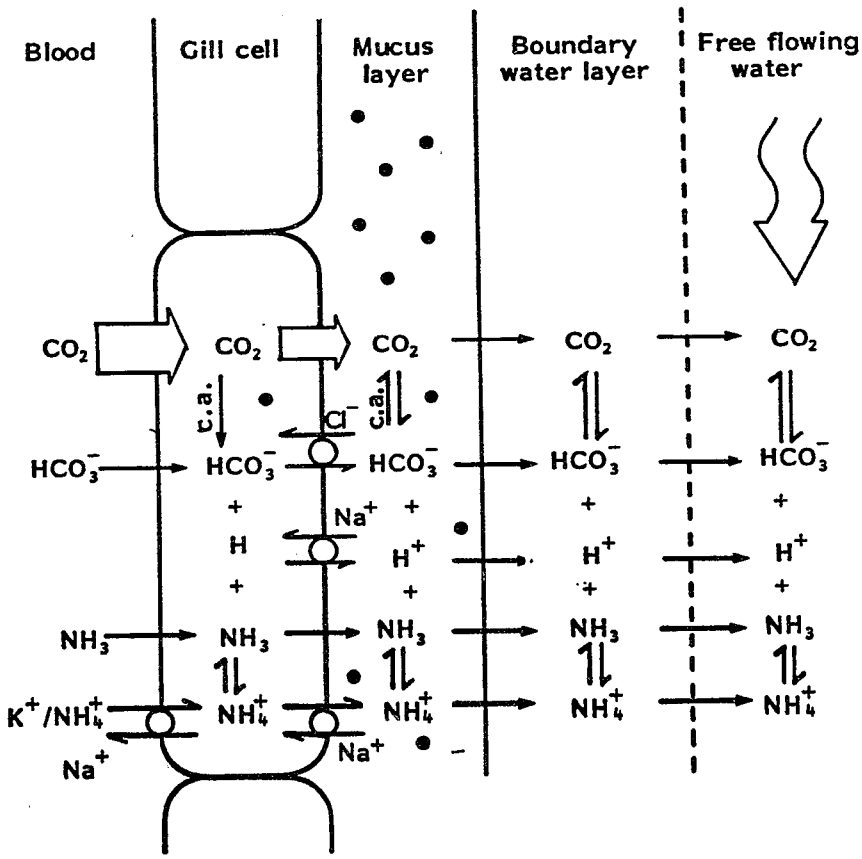

Figure 4. Schematic cross-section through the gill epithelium, mucus and water boundary layer, containing carbonic anhydrase (●). The thickness of the arrows denotes the approximate magnitude of the particular process illustrated (after Randall and Wright 1987).

layer of the gill (Wright et al. 1986). The extent of acidification will be determined by the rate of carbon dioxide excretion in relation to water flow and the buffering capacity and pH of the water. Excreted NH_3 will combine with a proton and increase water pH, but because more CO_2 than NH_3 is excreted across the gills, the overall effect usually will be an acidification of the boundary layer. NH_3 entering this acidified layer next to the gill surface will be converted to NH_4^+ and will diffuse out of the boundary water layer into the bulk medium.

The conversion of NH_3 to NH_4^+ and its subsequent diffusion from the boundary layer will reduce NH_3 levels in the water and, therefore, NH_3 levels in the blood (Wright et al. 1989). Thus, there is an interaction in the boundary layer between carbon dioxide and ammonia excretion. Starving trout have elevated blood NH_3 levels (Hillaby and Randall 1979) and this can be correlated with a decreased CO_2 excretion.

When water pH is below the apparent pK of the CO_2/HCO_3^- reaction, CO_2 excretion will have only a minor effect on water pH. At a water pH of 4.00, almost no HCO_3^- will form and, therefore, almost no further acidification of gill water will occur. In fact, the opposite will occur, for NH_3 excretion will now cause an increase in boundary water pH (Figure 5B). At a pH of 5.1, at 10°C, only 10% of the CO_2 entering the water will be converted to bicarbonate, but essentially all NH_3 will be converted to NH_4^+. It is at about this pH that there is no change in the pH of water as it passes over the gills (Figure 5B); that is, H^+ production with HCO_3^- formation is equal to H^+ consumption by NH_4^+ formation. This observation is consistent with a ratio of 10:1 for CO_2 to NH_3 excretion. The real situation is more complicated than indicated above, which ignores the contribution of $Na^+:NH_4^+$ and $Cl^-:HCO_3^-$ exchange to total carbon dioxide and ammonia excretion, as well as the excretion of any other compounds that may affect the pH of gill water.

Acid conditions reduce ammonia excretion because of an inhibition of $Na^+:NH_4^+$ exchange (Wright and Wood 1985) resulting in increased blood NH_3 levels. Acid conditions, in themselves, will favour NH_3 transfer because any NH_3 entering the water will be immediately converted to NH_4^+, maintaining NH_3 gradients across the gill epithelium. This effect, however, is not sufficient to offset the effect of inhibition of $Na^+:NH_4^+$ exchange, and blood NH_3 levels rise (Wright and Wood 1985). Under less acidic conditions (water pH 6.64), when there is no inhibition of $Na^+:NH_4^+$ exchange, blood NH_3 levels are reduced compared with fish exposed to water of pH 7.85 (Wright and Wood 1985).

The reverse is true under alkaline conditions. CO_2 excretion results in a large acidification of the boundary layer, as nearly all the CO_2 is converted to bicarbonate, or even carbonate, in the water (Figure 5B). This acidification, however, may not be sufficient to maintain NH_3 excretion, and alkaline conditions can lead to increased NH_3 levels in the body (Wright and Wood 1985, Randall and Wright 1989). Elevated ammonia levels stimulate urea production via uricolysis in some fish, but not trout (Olsen and Fromm 1971). Oreochromis alcalicus grahami lives in Lake Magadi, an alkaline lake, but unlike other teleost fish, is completely ureotelic, producing urea via the ornithine-urea cycle. Thus, this fish avoids problems of ammonia buildup when exposed to these very alkaline conditions by converting ammonia to the less toxic urea (Randall et al. 1989).

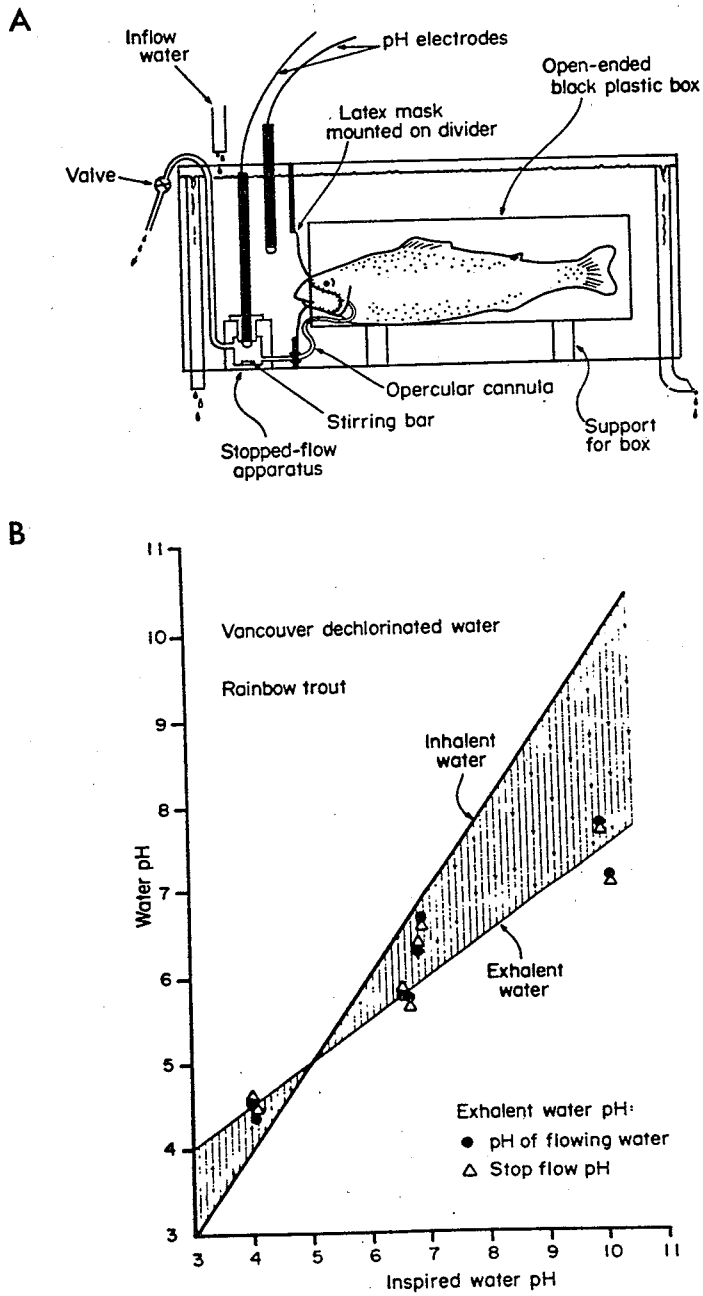

Figure 5. A--The apparatus for measuring inhalent and exhalent water pH in trout. The exhalent water transit time from the gills to the pH electrode was less than 2 seconds. The pH of the flowing water in the exhalent chamber was recorded and then flow was stopped and the equilibrium pH (stop flow pH) was measured. B--The relationship of exhalent to inhalent water pH in trout breathing dechlorinated Vancouver tapwater (from Randall and Wright 1989).

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CONTROL OF VENTILATION IN FISH

by
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INTRODUCTION

Respiratory gas exchange in fish takes place by diffusion over the surfaces of the gills. The gill arches are ventilated by muscular pumps around the mouth and pharynx at a rate determined by an oscillator in the CNS, which varies in its activity with oxygen supply and demand. The generation of the central respiratory rhythm and its modulation by peripheral mechanoreceptors and chemoreceptors are not completely understood. This review concentrates on some recent evidence of a role for circulating catecholamines in the control of the ventilatory response to hypoxemia.

RESPIRATORY RHYTHM GENERATION IN THE CNS

In fish, water is propelled continuously over the gills in one direction by the ventilatory muscles that operate around the jaws and skeletal elements in the gill arches, lining the pharynx (Ballintijn and Hughes 1965, Hughes and Ballintijn 1965). These muscles are innervated by cranial nerves with their neuron cell bodies located in the brainstem, close to the site of the central respiratory pattern generator (CPG).

Rhythmic ventilatory movements continue in fish following brain transection to isolate the medulla oblongata, although changes in pattern indicate that there are influences from higher centres (Shelton 1959). Central recording and marking techniques have identified a longitudinal strip of neurons with spontaneous respiration-related bursting activity, extending dorso-laterally throughout the whole extent of the medulla (Shelton 1970, Waldron 1972). These neurons comprise the trigeminal Vth, facial VIIth, glossopharyngeal IXth and vagal Xth motor nuclei, which drive the respiratory muscles, together with the descending trigeminal nucleus and the reticular formation (Ballintijn 1982). The respiratory rhythm is thought to originate in a diffuse CPG in the reticular formation, which remains functional following anaesthesia (Ballintijn 1988).

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All the motor nuclei are interconnected and each receives an afferent projection from the descending trigeminal nucleus and has efferent and afferent projections to and from the reticular formation (Figure 1). The intermediate facial nucleus, which receives vagal afferents from the gill arches that innervate a range of tonically and phasically active mechanoreceptors, projects to the motor nuclei (Ballintijn et al. 1983). Finally, areas in the mid brain such as the mesencephalic tegmentum have efferent and afferent connections with the reticular formation (Ballintijn 1982, 1988). These mesencephalic neurons are responsible for initiating bursts of ventilation during the periodic or bout respiration shown by some inactive or hyperoxic fish (Ballintijn 1988).

Studies of retrograde intraxonal transport of HRP along nerves innervating the respiratory muscles revealed that the neurons in the various motor nuclei are distributed in a sequential series in the brainstem of fish (Withington-Wray et al. 1986, Levings and Taylor 1987, Withington-Wray et al. 1988). Recordings of efferent activity from the central cut ends of the nerves innervating the respiratory muscles of the dogfish *Scyliorhinus canicula* (Barrett and Taylor 1985) and ray *Raia clavata* (E. W. Taylor and J. J. Levings, unpublished) have revealed that the branches of the Vth, VIIth, IXth and Xth cranial nerves fire sequentially in the order of the sequential rostro-caudal distribution of their moto-nuclei in the brainstem.

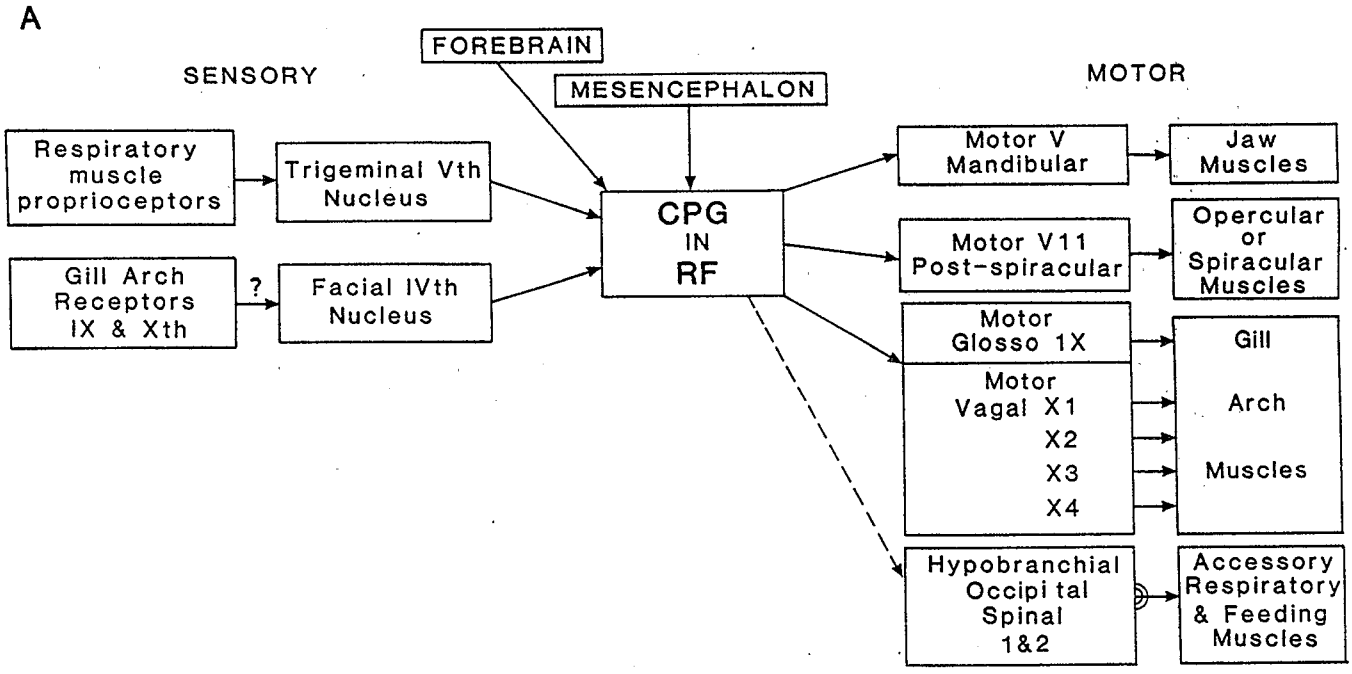

Figure 1. Summary diagram of the possible functional connections involved in the central nervous control of ventilation in fish. Abbreviations are: CPG, central pattern generator; RF, reticular formation.(Taken from Taylor 1989)

Experiments in which ventilatory movements were prevented with curare demonstrated that the CPG in fish continues to generate rhythmic bursts of activity that are slower than the unparalysed respiratory rhythm in teleosts (Ballintijn 1972) but faster in elasmobranchs (Barrett and Taylor 1985). These changes in rate following paralysis indicate that activity in the CPG normally is modulated by peripheral receptors. This is consistent with the fact that the CPG receives afferent information from a range of mechanoreceptors, proprioceptors and skin stretch receptors around the gill arches and jaws (Ballintijn 1982) with their sensory (afferent) innervation in cranial nerves V, VII, IX and X. Part of the population of motoneurons innervating the respiratory muscles is silent in the paralysed animal in both fish and mammals and may be stimulated to fire by artificially induced proprioceptive information (Ballintijn 1982). The recruitment of these silent motoneurons may serve to increase the motor output and consequent amplitude of contraction of respiratory muscles. In addition, fish may recruit feeding muscles innervated by the hypobranchial nerve trunk, which contains fibres from the occipital and anterior spinal motor nuclei (Figure 1). These insert on the skeletal elements around the mouth and pharynx and can contribute to active, forced ventilation when respiratory demand is high.

Thus, rhythmic contractions of the respiratory muscles are determined by a central respiratory pattern generator that is modulated by feedback on the force of ventilatory contractions from mechanoreceptors in the respiratory apparatus and by inputs from elsewhere in the CNS.

CHEMORECEPTOR RESPONSES

Fish pump water over their gills at rates controlled with respect to oxygen supply or demand. In teleost fish, ventilation increases when oxygen supply is reduced by environmental hypoxia, or when transport in the blood is reduced either directly by anemia or indirectly by hypercapnia, when the resultant acidosis causes a Root effect, reducing oxygen carrying capacity (for references see reviews by Randall 1982, Shelton et al. 1986). Similarly, an increase in oxygen demand during vigorous swimming or following the stress of experimental manipulation results in an increase in ventilation rate. Obversely, an increase in oxygen supply resulting from environmental hyperoxia causes a decrease in ventilation rate, which may result in hypoventilatory hypercapnia (Figure 2).

Most of these responses can be interpreted as arising from the stimulation of peripheral or central chemoreceptors sensitive to changes in oxygen supply (i.e., oxygen content and blood flow, Randall 1982). Evidence for the location and characteristics of the oxygen receptors is largely circumstantial and the mechanisms by which reflex ventilatory changes are initiated are not clear (Taylor 1985). Both the ventilatory (Eclancher and Dejours 1975) and cardiac (e.g., Butler and Taylor 1971) responses to sudden exposure to hypoxia or exercise are rapid in onset indicating that nervous pathways are involved. The motor arm of the response is of course nervous as the ventilatory muscles are innervated by the efferent cranial nerves (Hughes and Ballintijn 1978). The afferent arm has not been clearly identified (Taylor 1985) and neuropharmacology of the central connections implicated in these reflex responses, and the possible roles for circulating hormones are not known. Circulating catecholamines may be involved in the control of ventilation and this review now concentrates on this point.

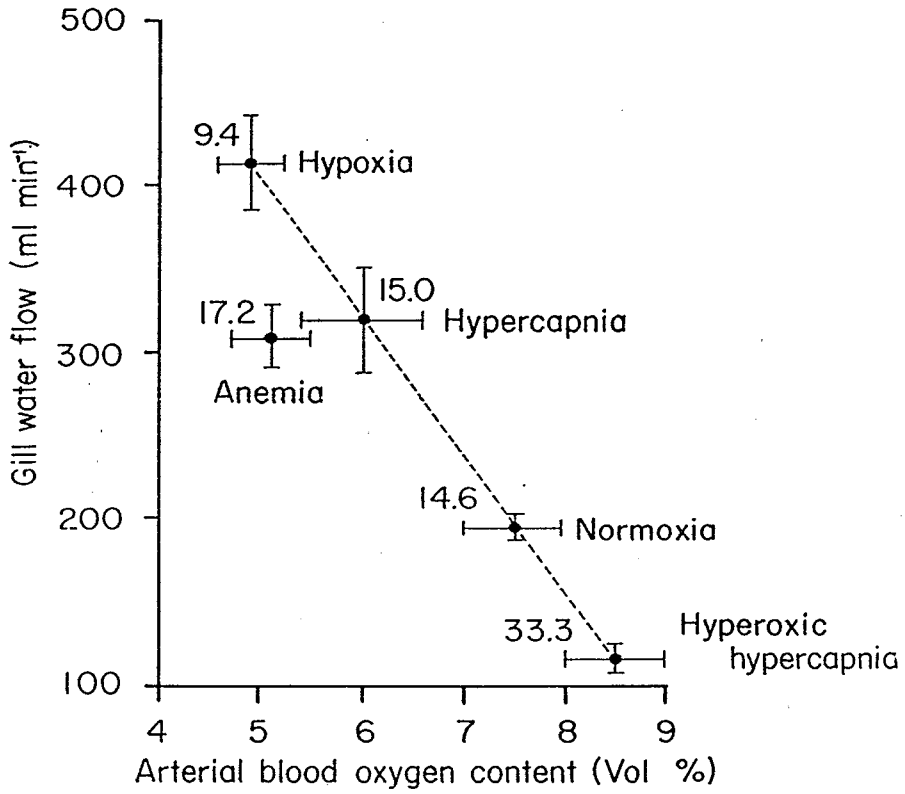

Figure 2. The relationship between rate of water flow over the gills and the oxygen content of arterialised dorsal aortic blood from the rainbow trout Salmo gairdneri (mean values ± SE). The number by each point is the corresponding arterial oxygen partial pressure in kPa. (Taken from Randall 1982)

BLOOD CATECHOLAMINE LEVELS

Teleost fish have low (<0.5 nmol l^{-1}), but measurable, resting levels of circulating catecholamines (Boutilier et al. 1988). The levels are higher in the elasmobranch fish *Scyliorhinus canicula* (above 20 nmol l^{-1}), possibly because these fish lack a sympathetic connection to the heart and branchial (gill) apparatus, which may enhance the role of circulating catecholamines in cardiovascular and ventilatory control (Butler et al. 1978). Changes in physiological state, particularly those induced by stressful stimuli such as physical disturbance (Nakano and Tomlinson 1967) cause an increase in circulating catecholamine levels. Other stimuli include hypoxia (Boutilier et al. 1988), anemia (Iwama et al. 1987), hypercapnia (Perry et al. 1988), acid infusion (Boutilier et al. 1986), and violent exercise (Primmett et al. 1986).

Circulating catecholamines play a number of important roles in the control of metabolism and respiratory gas exchange and transport, including

stimulation of anaerobic glycolysis during exercise; increased contractility of the heart, vasodilation or vasoconstriction of peripheral blood vessels, with resultant changes in blood pressure and vascular resistance to blood flow (Wood 1975); increased permeability of the gill respiratory epithelium (Isaia et al. 1978); release of red blood cells from the spleen, increased red cell volume and intracellular pH (Nikinmaa 1982). In addition, it is now clear that many stimuli causing ventilatory increases also are accompanied by changes in circulating catecholamines (Figure 3). The simplest interpretation of this observation is that the ventilatory changes are in some way induced or reinforced by changes in circulating catecholamine levels.

CATECHOLAMINES AND VENTILATION

Measurable increases in circulating catecholamine levels in fish are associated with environmental hypoxia in both teleosts (Boutilier et al. 1988) and elasmobranchs (Butler et al. 1978). The hypoxemia resulting from anemia (Iwama et al. 1987) and hypercapnia (Perry et al. 1988) also is associated with increased catecholamine levels. Thus many stimuli that result in increases in ventilation (Figure 2) also cause increased levels of circulating catecholamines (Figure 3).

Evidence for a direct link between ventilatory responses and changes in catecholamine levels have been obtained by acid infusion (K. Holmgren, unpublished observations) and hypoxia (S. Aota, unpublished observations) in trout. Both cause an increase in circulating catecholamines and in gill ventilation.

The β-adrenergic blocking agent, propranolol, inhibits the ventilatory response but not the increase in blood catecholamines. During hyperoxia, acid infusion did not cause an increase in circulating catecholamines nor was there any increase in gill ventilation. The simplest explanation of these data is that the increase in ventilation during hypoxia or following an acid injection is mediated by the release of catecholamines into the circulation. The site of action of these catecholamines could be either peripheral or central, but the pathway is blocked by propranolol and so involves β-adrenergic receptors.

SITE OF ACTION OF CATECHOLAMINES

Peyraud-Waitzenegger (1979) showed that intravenous injection of adrenaline in the eel caused an increase in ventilation. The injection of catecholamines into dogfish (*Scyliorhinus canicula* and *Squalus acanthius*) caused changes in central respiratory drive measured as the efferent, motor activity in a branchial branch (to a gill arch) of the vagus nerve of paralysed fish (i.e., in the absence of peripheral mechanoreceptor inputs). The fish were force ventilated with hyperoxic seawater to reduce stress and decrease endogenous levels of circulating catecholamines (E. W. Taylor and D. J. Randall-unpublished observations). Intravenous injection of adrenaline or noradrenaline, after a short delay (20 sec), caused a transient but marked increase in efferent activity in *S. canicula*, which recovered to normal activity, then after a slightly longer delay showed a further transient increase.

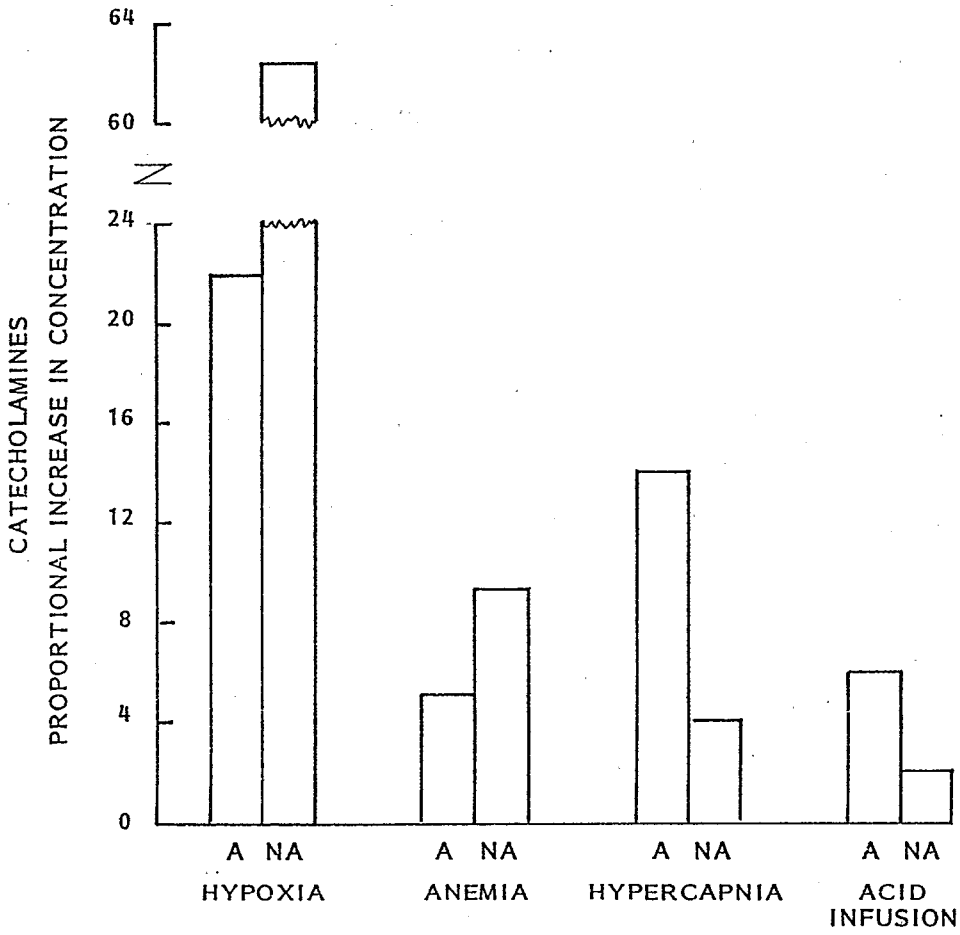

Figure 3. The proportional increase in the plasma levels of circulating catecholamines in the trout Salmo gairdneri elicited by exposure to hypoxia or hypercapnia by experimentally induced anemia and by acid infusion. Abbreviations are: A, adrenaline; NA, noradrenaline.

Our interpretation of this rather complex response is that the bolus of blood containing high levels of injected catecholamines passed for two circuits of the circulatory system over a receptive area that triggered an increase in activity in the medulla. Subsequently, the catecholamines would be extracted from the blood and metabolized by the gills and other tissues (Nekvasil and Olson 1986a). The approximate location of the receptive area can be deduced from our limited knowledge of circulation times in the dogfish to lie efferent to the heart, possibly in the cerebral circulation. These effects of intravenous injection of high concentrations of catecholamines were most marked for noradrenaline. This may cross the blood-brain barrier in fish more easily than adrenaline (Nekvasil and Olson 1986 b).

When catecholamines were injected into curarised, hyperoxic *S. canicula* in which activity in a hypobranchial nerve, which innervates feeding muscles,

was recorded simultaneously with that in a branchial branch of the vagus, direct evidence of nervous recruitment was observed (J. J. Levings and E. W. Taylor, unpublished observations). In the hyperoxic fish prior to injection of adrenaline, the branchial branch showed regular bursting activity, but the hypobranchial nerve showed low levels of activity and only fired intermittently, with a respiration-related activity. Injection of adrenaline caused high levels of respiration-related, bursting activity in the hypobranchial nerve, which resembled or exceeded that observed in the disturbed normoxic and fictively hyperpnoeic fish. This result seems to identify a role for circulating catecholamines in the onset of forced ventilation.

We currently are investigating the possibility that catecholamines exert their effects upon the central pattern generator or the respiratory motoneurons by direct injection of catecholamines into the CNS. Injection of small volumes (8-20 μl) of a 10^{-4} molar solution of adrenaline into the fourth ventricle of *S. acanthius* caused a marked change in the pattern of central respiratory drive (Figure 4). The response was complex but stereotyped. An initial increase in the rate of bursting of respiratory motor units was followed by a slowing of the rhythm accompanied by a huge increase in the activity within each burst (Figure 4b), apparently due to the recruitment of units having larger recorded spikes, implying larger fibre diameters, as all vagal fibres are myelinated (Short et al. 1977). This implies that the increase in ventilation brought about by injection of catecholamines may result from an increase in stroke volume rather than rate of contractions of the respiratory apparatus. The response to injected catecholamines was blocked by simultaneous injection of propranolol, a β-adrenergic antagonist (Figure 4c).

These data imply that areas in the CNS, accessible from the fourth ventricle, respond to an increase in catecholamine concentration with an increase in central respiratory drive. The vagal respiratory motoneurons lie in the dorsal vagal motor nucleus, which is situated bilaterally in the medulla in a medial position close to the wall of the fourth ventricle (Withington-Wray et al. 1986). These neurons are rhythmically active, under the influence of the central pattern generator, and supply the efferent innervation to intrinsic respiratory muscles in the gill arches. Their location close to the wall of the fourth ventricle may have caused them to be directly affected by the injection of catecholamines into this location. Identification of the site of action of catecholamines on the respiratory rhythm generator and associated respiratory motor neurons in the medulla must await microelectrode studies of single, identified neurons.

CONCLUSIONS

Our current knowledge of the control of ventilation in fish is incomplete at all levels. The respiratory rhythm originates in a medullary central pattern generator, which has yet to be clearly identified and characterised. Its activity is modulated by inputs from elsewhere in the CNS and from peripheral mechanoreceptors. The central location of respiratory motor neurons, innervating the various respiratory muscles, has been described in detail for some fish, particularly elasmobranchs.

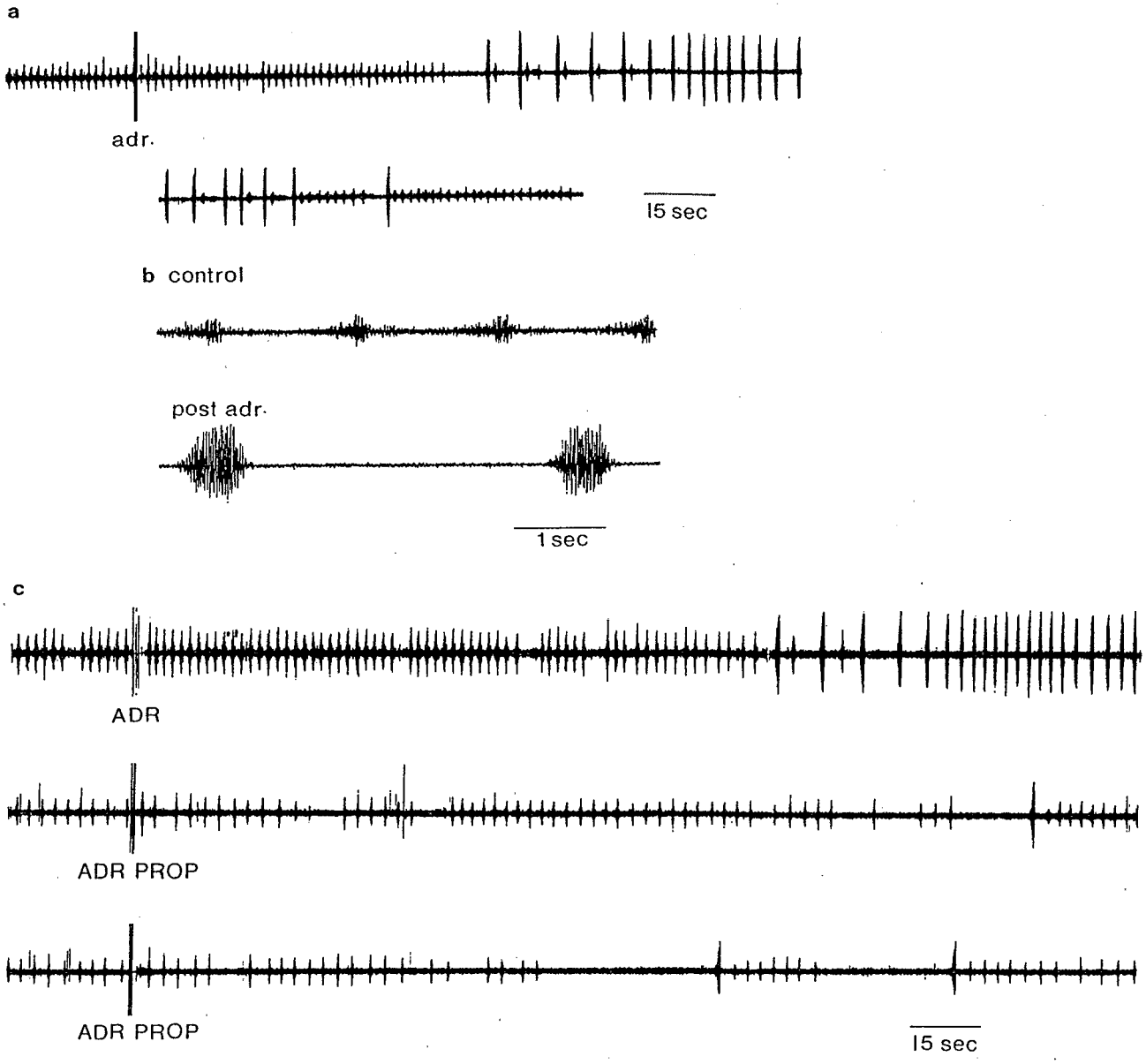

Figure 4. The effect of injection of adrenaline into the fourth ventricle of the dogfish Squalus acanthius upon efferent activity in the third branchial (respiratory) branch of the vagus nerve. a--injection of 20 ul of 10^{-4} molar adrenaline (adr.), after a 20-sec latent period, induced a transitory increase in the frequency of the bursting activity recorded from the nerve. After about 75 sec, this was replaced by a slowing in bursting rate accompanied by an increase in the amplitude of the recorded bursts. b--detail of recording from trace in (a) to compare the rate and amplitude of the bursts recorded before and after injection of adrenaline. c--injection of adrenaline (ADR) into the fourth ventricle of another experimental animal induced a similar response to (a) that was subsequently blocked by simultaneous injection of adrenaline and propranolol (ADR PROP).

We are still unclear, however, about the link between the CPG and the sequential firing of the motor neurons, which result in coordinated contractions of the respiratory muscles, and about the mechanisms that result in recruitment of feeding muscles into forced ventilation. Ventilation is matched to oxygen requirements by stimulation of chemoreceptors, which seem to respond to oxygen content or supply. The precise location and characteristics of these chemoreceptors are still not known.

Chemoreceptor stimulation evokes a number of reflex changes in the respiratory and cardiovascular systems of fish that are rapid in onset and seem adaptive (e.g., increased ventilation and a bradycardia in response to hypoxia). Conditions that result in hypoxemia and the consequent ventilatory changes also cause an elevation in circulating catecholamine levels. We have explored the possibility of a causal relationship between these levels and the ventilatory response. Strong evidence arises from experiments on hypoxia and acid infusion, which trigger a ventilatory increase and a rise in circulating catecholamines. Both ventilatory responses are blocked by an injection of propranolol despite an increase in catecholamine levels.

The ventilatory response to hypoxia, at least, occurs very rapidly, perhaps before any marked increase in circulating catecholamines and almost certainly before any blood catecholamines could reach the respiratory neurons. This argues for an immediate neuronal reflex based on chemoreceptors in the gill region responding to hypoxia. Clearly, circulating catecholamines also affect ventilation through some action in the medulla and could act in concert with a direct neuronal chemoreceptive drive during hypoxia. The studies on acid infusion during hyperoxia, where there is an acidosis but no increase in ventilation or blood catecholamines, would argue against any hydrogen ion receptor, either peripheral or central, being involved in the reflex ventilatory response to acidotic conditions in fish.

The release of catecholamines into the circulation, therefore, seems to be an absolute requirement for the ventilatory response to acidosis in fish. Present evidence supports a role for β-adrenergic receptors on respiratory neurons, stimulated by changes in the levels of circulating catecholamines, in the control of ventilatory responses to changes in blood oxygen levels in fish.

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ACCUMULATION OF CARBON DIOXIDE IN FISH FARMS WITH RECIRCULATING WATER

by
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INTRODUCTION

In northern Europe, intensive culturing of eels, Anguilla anguilla, in heated fresh water at 25°C is economically feasible in recirculated systems provided with mechanical and biological filtration. Because of the consumption of energy for heating, the eels are grown in tanks with recirculating water, located in well-insulated buildings. The culture is based on weaning of wild glass-eels to dry feed.

The density of fish is high (75 to 150 kg/m^2) in eel farms with recirculating water. Because the eels are fed continuously and the water is warm, the oxygen consumption of the fish is high. Some fish are reported to grow slower at hypoxic conditions (pO_2 = 70 mmHg) (Stewart et al. 1967). Hence, it is common practice to enrich the water supplying the fish tanks with pure oxygen (to 200 to 400% O_2 saturation) to ensure that the eels are never exposed to hypoxic water.

In a typical eel farm with recirculating water (Figure 1), normoxic conditions in the fish tanks is fulfilled by injecting pure oxygen, but carbon dioxide produced by the fish is not removed effectively. Because high carbon dioxide concentration is reported to inhibit oxygen consumption, to reduce the oxygen affinity and the oxygen carrying capacity of blood (Root 1931), or even to induce a narcotic acidicosis, it may be profitable to focus on reducing carbon dioxide in recirculating eel farms.

THE EEL FARM

A diagram of a typical Danish eel farm is shown in Figure 2. From the fish tanks, the water flows through an over-flow to a sludge separator and pump sump. The water then is pumped through an aerator to submerged and trickle filters for nitrification and denitrification. Finally the water is pumped through an oxygenator, supplied with pure oxygen, and back to the fish tanks. In the figure, the measured pH and partial pressures of oxygen (pO_2) are indicated, the latter in mmHG. Water is maintained at 6.8 to 7.2 pH by adding bicarbonate or another base. Danish ground water has a high bicarbonate concentration and a relatively high CO_2 concentration.

It appears that the oxygen tension is normoxic or hyperoxic in the entire system. It also appears that carbon dioxide tension is considerably

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Production	:	100 ton/year
Standing stock	:	50 ton
Number of fish tanks	:	50
Area of fish tanks	:	850 m2 (4.5 m dia.)
Volume of fish tanks	:	425 m3
Total vol. of system	:	650 m3
Recirculation flow	:	650 m3/hour
Density of fish	:	50 - 100 kg/m2
Food - dry pellets	:	550 kg/day (1.1 % b.w. pr. day)
Water oxygen saturation	:	300 % at inlet - 100 % at outl
Oxygen supply	:	300 kg/day
HCO3- supply	:	75 kg/day
Production time	:	24 months (from .35 to 250 g)
Temperature	:	25 DegC
Area of filter	:	25000 m2
Current market value	:	8 US$/kg

Figure 1. Typical eel farm with recirculating water.

elevated, hypercapnic, in the entire system, even though some CO_2 is "blown off", especially at the trickle filter. A cCO_2 of 32 mmHg. For comparison, CO_2 in the normal eel habitat rarely exceeds 5 mmHg. Values of 20 to 30 mmHg are far above what eels normally are exposed to.

THE EEL AND THE BLOOD

Because the excretion of carbon dioxide over the gills is a passive diffusion process, pCO_2 in the blood is somewhat higher than in the ambient water. Blood pCO_2 in eels in natural conditions is 3 to 6 mmHg. In the fish farm with pCO_2 = 32 mmHg in the fish tank, the blood pCO_2 must be even higher.

A pCO_2 above 30 mmHg for fish is far higher than normal. An increase in CO_2 will cause a decrease in pH, which in fish will be compensated for by an increase in the blood bicarbonate concentration. The coherence between pH, pCO_2, bicarbonate concentration, and total CO_2 concentration is shown in the Davenport diagram in Figure 3.

Assuming a pCO_2 of 5 mmHg and pH = 7.7 as normal values for eel, bicarbonate concentration equals 10 meq/l, according to Figure 3. In the eel farm situation, however, with a pCO_2 of 32 mmHg, the blood will have a slightly lower pH. At pH = 7.5 and 7.4, bicarbonate concentration will be 37 and 29 meq/l, respectively. These bicarbonate concentrations are far higher than what hitherto has been considered physiologically normal for fish.

Heisler (1984 1986) suggested that the maximal blood HCO_3- concentration in hypercapnic fish, which can be used for compensating a hypercapnic acidosis, is 30 $mmol^{-1}$. Recently, however, Dimberg (1988) reported bicarbonate concentrations of 55 to 66 $mmol^{-1}$ in rainbow trout exposed to hypercapnic water with a pCO_2 of 26 to 34 mmHg.

CONCLUSION

Carbon dioxide has long been known to reduce the affinity of blood for oxygen (Root 1931), to reduce the oxygen carrying capacity of the blood, and to inhibit the metabolic rate of fish (Basu 1959, Saunders 1962, Beamish 1964). Basu reported that the active oxygen consumption of several freshwater fishes decreased with increasing CO_2. At higher concentrations, CO_2 depresses ventilation and can even induce a narcotic acidosis (Dejours 1988).

Only limited information is available on the effect of CO_2 and oxygen on swimming capacity. Dahlberg et al. (1968) reported that the prolonged

swimming speed of largemouth bass did not decrease in response to CO_2 concentrations up to 48 mg l^{-1}. The performance of coho salmon, on the contrary, decreased with increasing CO_2 concentrations between 2 and 61 mg l^{-1}. The effect of CO_2 was especially pronounced at high concentration of oxygen (10mg l^{-1}). This decrease of performance may be a result of the decrease in blood oxygen affinity and carrying capacity, or an increased cost of acid-base regulation.

In spite of the unnaturally high CO_2 tensions in the fish-farms, the eels seems to be in good health and growing well. It is likely, however, that these extreme CO_2 tensions have some consequences for the well-being of the eels. Even though the fish are growing well, they may grow even better at more normal CO_2 conditions, if, for example, less energy has to be used for acid-base regulation. In one fish farm, it was observed that the eels appetite increased after installing a trickle filter.

The results of future research (growth studies, acid-base balance, etc.) will determine whether more effort should be aimed at decreasing the CO_2 levels in intensive fish farms with recirculating water and oxygenators.

If it is desired to get more natural CO_2-conditions, it is not sufficient to adjust the water pH by supplying bicarbonate, as practiced in many fish farms. Only by increasing the contact between the water and the

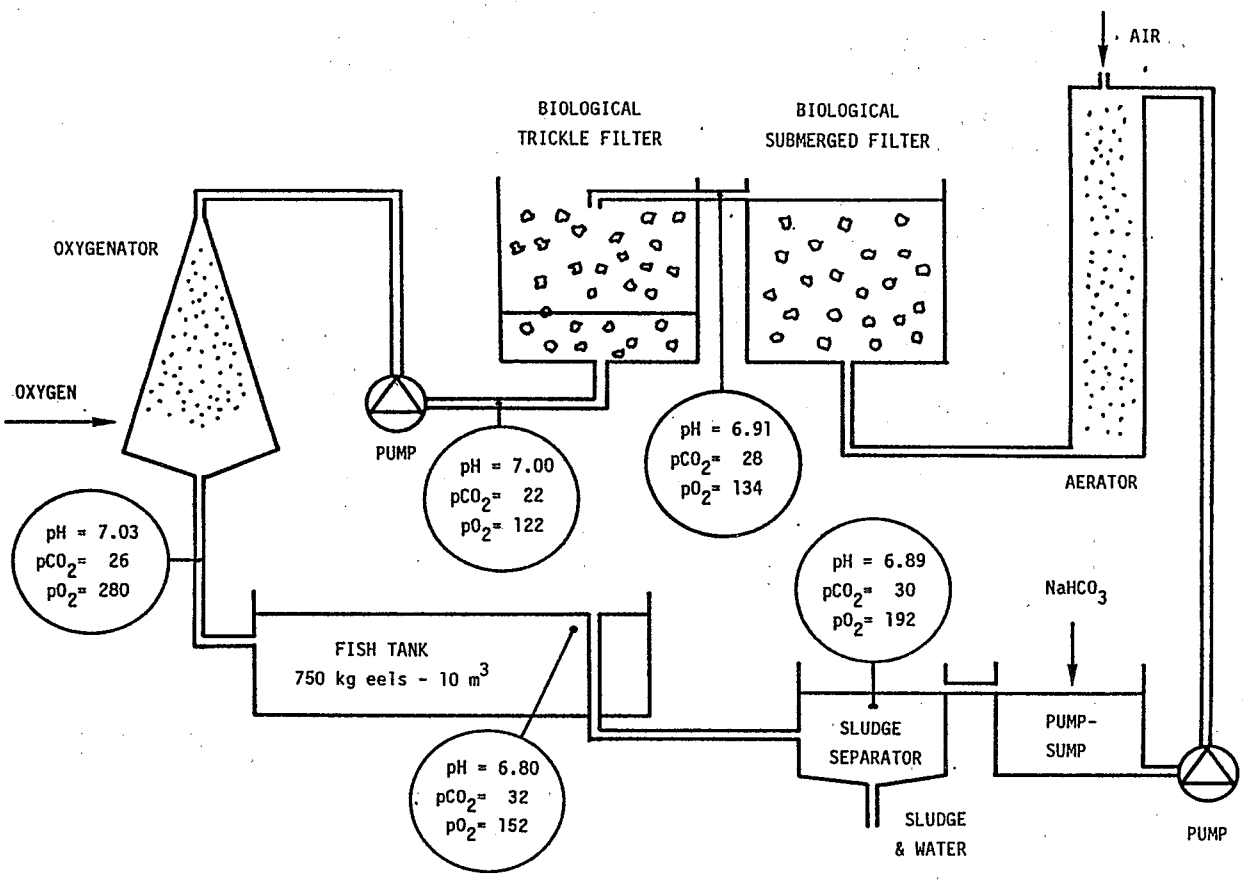

Figure 2. Diagram of eel farm with recirculating water.

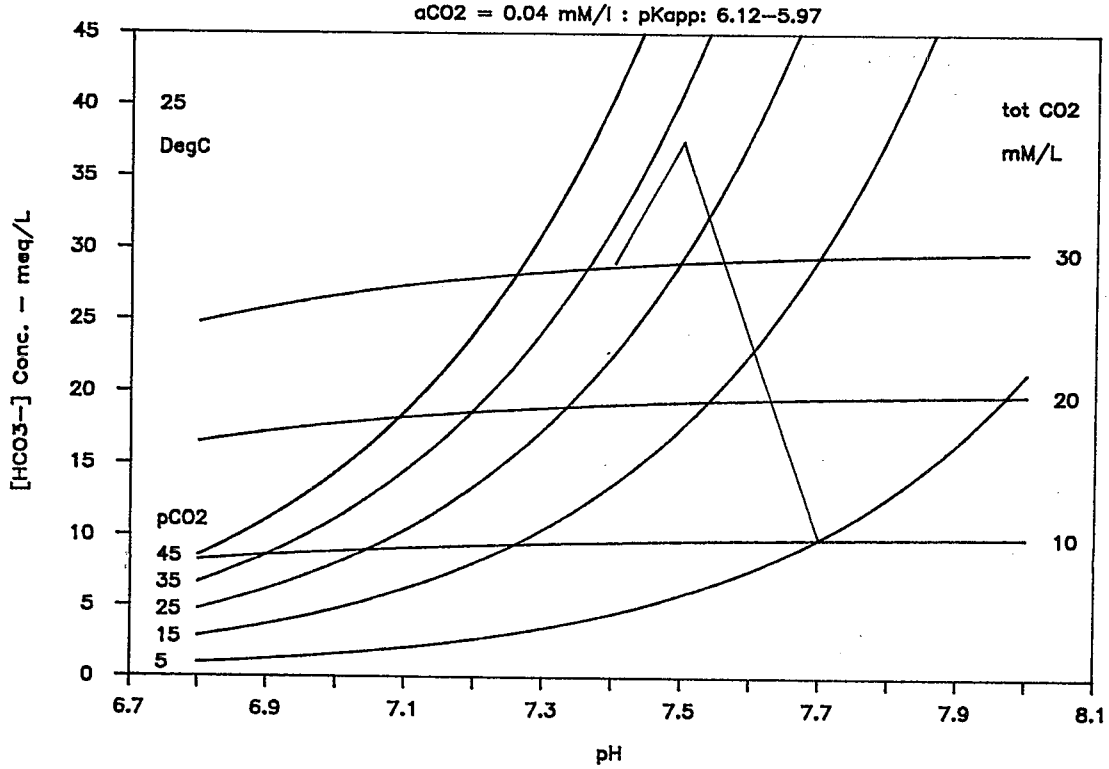

Figure 3. Davenport diagram.

atmosphere, for example with trickle filters, can the diffusion of CO_2 from the water to the atmosphere be facilitated, hence decreasing the CO_2-tension in the water.

ACKNOWLEDGMENT

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IMPACT OF ENVIRONMENTAL ACIDIFICATION ON GILL FUNCTION IN FISH

by

Chris M. Wood[1] and D. Gordon McDonald[1]

INTRODUCTION

Although environmental acidification resulting from industrial and domestic SO_2 and NO_x emissions has been documented most strongly in north-eastern North America and northern Europe, it is now recognized as a global problem. Zhao and Sun (1986) have reported that much of China to the south of the Yangtze River is subject to "acid rain" due to a combination of intensive combustion of high-sulphur coal and low atmospheric buffer capacity (i.e., low ammonia and alkaline particulates). Soils are generally acidic, and some major cities report annual rainfall pHs as low (~4.0) as those in the most seriously affected areas of eastern Canada and Scandinavia.

Damaging effects on freshwater fisheries may occur anywhere in the world where precipitation pH is persistently below 5.6 (the pH of distilled water in equilibrium with atmospheric CO_2) and the buffer capacity of the watershed is low. Such "softwater" regions are generally characterized by low calcium carbonate alkalinity in soils, bedrock, sediments, and the water column itself. The softwater pH range of environmental significance to fish is 4.0 to 6.0; pHs below 4.0 rarely, if ever, occur. Most fish populations are not adversely affected at pHs above 6.0.

Within this range, the deleterious effects of acidity are complex, and include trophic degradation, reproductive failure, early life stage mortality, and lethal and sublethal toxicity to juvenile and adult fish (Howells 1984). Over the past decade, our research program has concentrated on this latter area, investigating the physiological mechanisms of acid toxicity. The present paper provides an overview of this research, with emphasis on recent findings in the area of combined acid and aluminum toxicity, sublethal effects, and acclimation.

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PHYSIOLOGICAL RESPONSES TO PURE ACID STRESS AND THE INFLUENCE OF CALCIUM

Figure 1 summarizes the major effects of acute exposure to pH = 4.0 - 4.5 on salmonids such as the rainbow trout (Salmo gairdneri). For detailed reviews of this earlier work on the lethal effects of acid alone, the reader is referred to Wood and McDonald (1982), McDonald (1983), and Wood (1988). At this pH range, disturbances in respiratory gas exchange do not occur, although they may contribute to toxicity when metals are present (see below) or at more acidic pHs where branchial epithelial swelling, delamination, and mucification may occur. Nevertheless, there are multiple toxic actions, including ionic dilution and acidification of the blood and extracellular fluids (ECF), demineralization of bone, electrolyte losses from the intracellular fluids (ICF), especially white muscle, and disturbances of renal function.

By far the most important toxic effects, and those ultimately responsible for the death of the fish, however, are exerted on the external surface of the gills. This is a compound action, comprising an inhibition of active Na^+ and Cl^- uptake through the mitochondrial-rich "chloride cells," and perhaps more importantly, a stimulation of passive diffusive losses of Na^+, Cl^-, and other electrolytes. The latter is thought to result from an increased permeability of the paracellular diffusion channels in the branchial epithelium. The net result is a large loss of electrolytes from the blood plasma, and secondarily from the tissues. Osmotic pressure falls faster in the ECF than in the ICF, and in compensation, water shifts from plasma into cells, especially muscle and erythrocytes. Hematocrit, plasma protein concentration, and blood viscosity all increase markedly as the red blood cells swell and the plasma volume contracts. These effects are compounded by adrenergically mediated vasoconstriction of systemic resistance vessels, cardioacceleration, and mobilization of additional red cells from the spleen. In the end, greatly elevated arterial blood pressures, hematocrits as high as 70%, and plasma volumes as low as half normal values result in a circulatory failure ultimately attributable to the branchial ion loss.

In laboratory tests, elevations in water calcium protect against these ion losses, and therefore against acid toxicity, an observation that is confirmed by a wealth of field data (e.g., Wright and Snekvik 1978, Howells et al. 1983). The mechanism of this protection is thought to be the well known effect of Ca^{2+} in limiting membrane permeability, specifically in "plugging" the branchial paracellular diffusion channels that are permeabilized by H^+. Those natural softwaters most sensitive to acidification due to their low alkalinity, of course, are also low in calcium (generally in the range 25 - 400 uequiv/L = 0.5 - 8 mg/L), which exacerbates the problem for the fish. (Waters of higher calcium content are rarely acidified, except in cases of mine acid drainage.) The ameliorative effects of "liming," therefore, are due to the increases in water calcium, as well as in pH and alkalinity.

A point of considerable confusion is the often-stated view that increased water calcium also should protect the fish against H^+ entry and internal acidosis. This is clearly untrue, as illustrated by the data in Figure 2. Indeed at a given acidic pH, the higher the water calcium level, the greater is the depression in arterial pH and metabolic acidosis, whereas

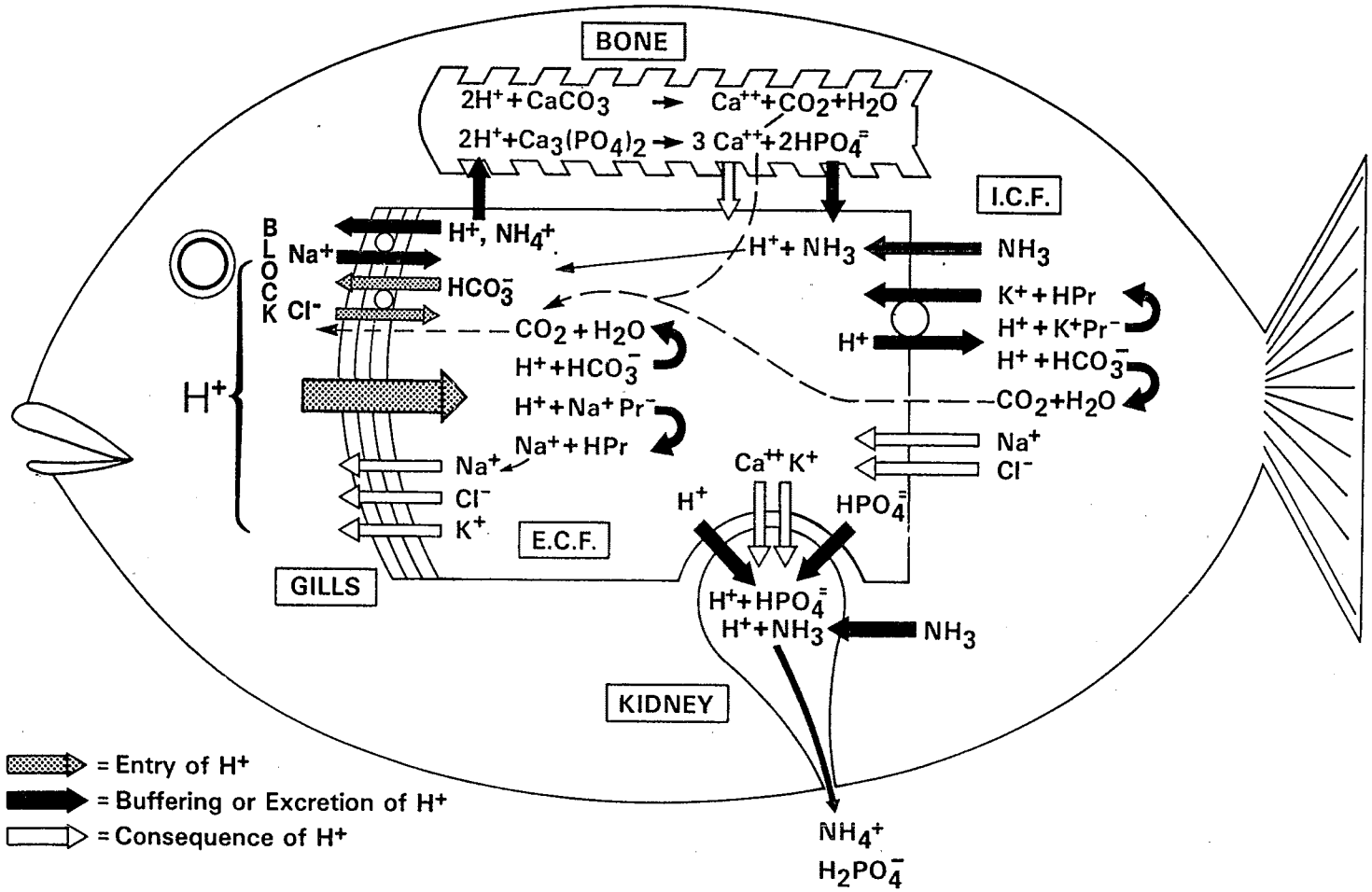

Figure 1. Summary of the major physiological effects of acute exposure to pH = 4.0 to 4.5 (pure acid stress) in salmonid fishes (modified from Wood and McDonald 1982).

the smaller is the plasma Na^+ and Cl^- deficit. This pattern has been confirmed by the direct measurement of ion and "acidic equivalent" (net H^+) fluxes across the gills.

The explanation for this apparent dichotomy lies in simple physical principles--the laws of electrical neutrality and the "strong ion difference" relationships (Stewart 1983). Stated very simply, the flux of strong cations (mainly Na^+ plus K^+) minus the flux of strong anions (mainly Cl^-) across a biological membrane such as the gill will automatically constrain an equal "flux" of acidic equivalents in the opposite direction. Water is an infinite source or sink of acidic equivalents; net H^+ flux is not an independent variable, but rather one dependent upon differential strong cation and anion flux. Any resultant acid-base disturbance in the fish, therefore, is an unavoidable consequence of ionoregulatory disturbance.

Higher water calcium levels protect against strong electrolyte losses at low pH, but are more effective in limiting Cl^- efflux than Na^+ or K^+ efflux, probably because the diffusion channels become cation selective. At lower calcium levels representative of natural softwaters, overall electrolyte permeability increases, but anion permeability catches up to and may eventually exceed cation permeability. As a result, there is negligible net H^+ uptake, or even a slight net H^+ efflux across the gills. Internal acid-base status is unchanged or may even become slightly alkalotic (cf. Figure 2).

In summary, under pure acid stress, at environmentally realistic levels of pH (4.0 - 6.0) and calcium (25 - 400 uequiv/L), toxicity is due to ionoregulatory disturbance, not to acidosis, and not to respiratory failure.

THE IMPORTANCE OF ALUMINUM

Unfortunately, pure acid stress itself may not be an environmentally realistic situation. An increasing body of evidence argues for the involvement of aluminum in acid toxicity in the field (e.g. Schofield and Trojnar 1980, Harvey and McArdle 1986, Johnson et al. 1987). Aluminum is the third most abundant element in the earth's crust, occurring ubiquitously in rocks, soil, and sediments. Its solubility increases exponentially as pH falls below about 5.6 (Figure 3); consequently, it is readily mobilized into acidified softwaters (Driscoll 1980, Lazerte 1984). This mobilization has been repeatedly suggested as the "missing link" explaining why mortality in the field is often much greater than that in the laboratory at the same pH and calcium levels, although this point remains controversial (e.g. Muniz and Leivestad 1980, Schofield and Trojnar 1980, Howells et al. 1983, Howells 1984, Neville 1985, Schindler 1988).

Much of the uncertainty surrounding the role of aluminum devolves from its very complex aqueous chemistry. A highly simplified speciation model is shown in Figure 3, which deliberately omits the fluoride, sulphate, carbonate, and organic anion binding reactions, all of which are thought to lessen aluminum toxicity, and the various polymerization reactions that may occur under super-saturated conditions. The equilibria plotted are based on the dissolution of microcrystalline gibbsite, which is probably more representa-

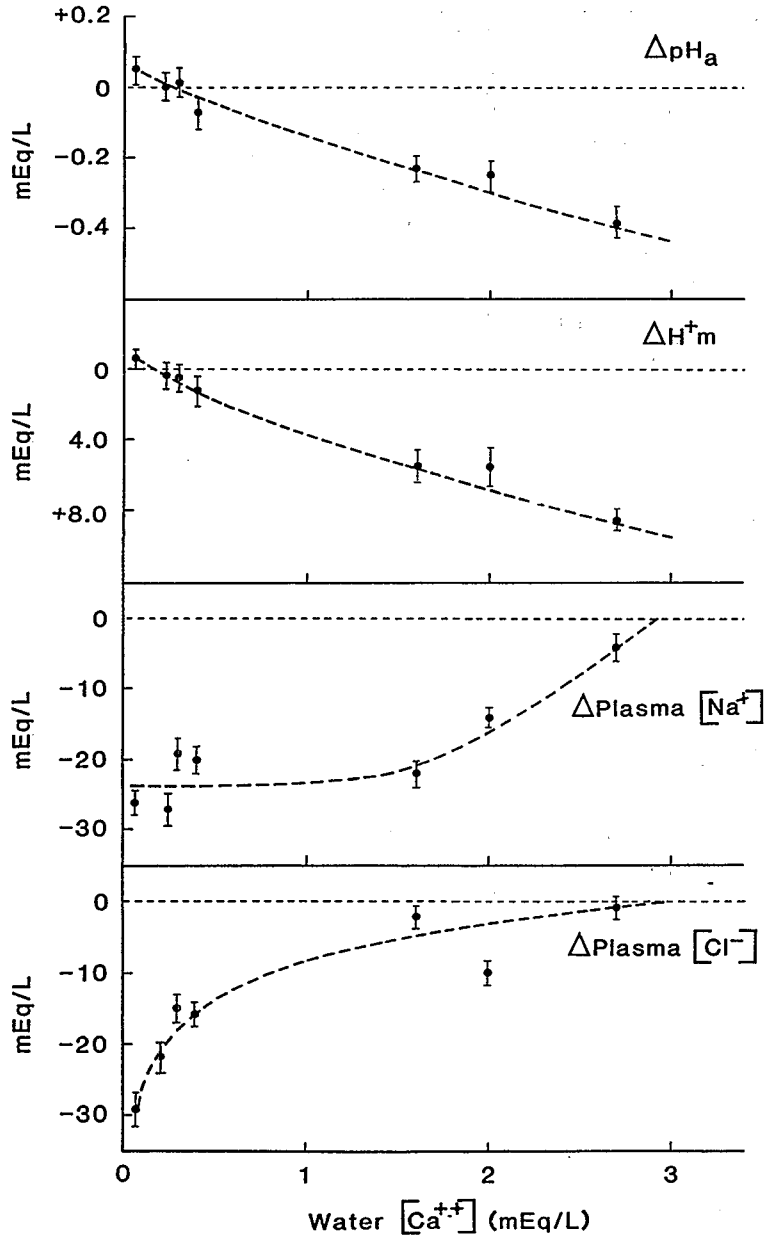

Figure 2. The relationship between water calcium concentration and the extent of various acid-base and ionic disturbances in the arterial blood of rainbow trout exposed to pH 4.3 for 3 days. (A) Changes in arterial pH. (B) Changes in blood metabolic acid load. (C) Changes in plasma sodium concentration. (D) Changes in plasma chloride concentration. Means ±1 SEM (n = 4 - 24). (from Wood 1988)

tive of field conditions (at least in eastern Canada, Lazerte 1984). A total solubility curve based on amorphous aluminum hydroxide also is presented. The latter is representative of freshly prepared solutions used in the laboratory. Figure 3 illustrates that the speciation, as well as the solubility, of inorganic monomeric aluminum is critically dependent upon pH. As pH falls, the non-toxic anion $Al(OH)_4^-$ is sequentially replaced by the $Al(OH)_2^+$, $Al(OH)^{2+}$, and finally Al^{3+} cations. All these cationic forms are thought to be toxic to fish, although Al^{3+} could also be protective through its ability to mimic the action of Ca^{2+} (Baker and Schofield 1982). Much of the previous work on the physiological responses of fish to aluminum has been done in recirculating systems, often without precise control of pH or water chemistry, or realistic water calcium levels. The resulting divergence in conclusions may well reflect changing solubility, speciation, organic and inorganic complexation, and calcium/aluminum interactions.

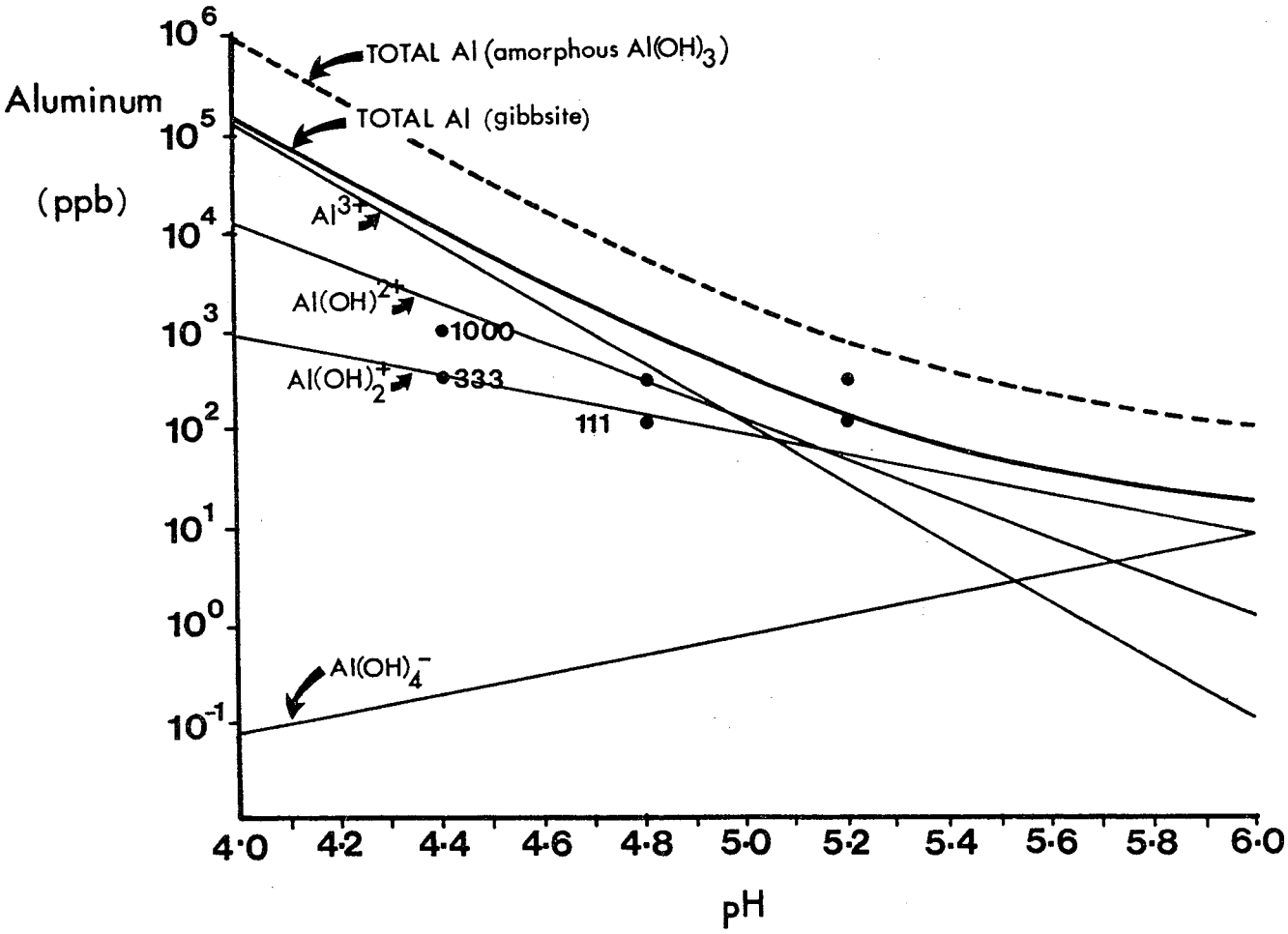

Figure 3. Speciation chemistry and solubility of aluminum (omitting complexation reactions) as a function of pH in low ionic strength waters. The Al/pH combinations tested in the present study are noted.

PHYSIOLOGICAL RESPONSES TO ACID/ALUMINUM/CALCIUM COMBINATIONS

The goal of our own recent work has been to study the physiological responses of juvenile and adult salmonids to various environmentally representative combinations of pH (4.4, 4.8, 5.2), calcium (25, 400 uequiv/L), and aluminum (0, 111, 333, 1000 ug/L) under defined artificial softwater conditions (reverse osmosis water, 50 uequiv/L Na^+, 0 DOC and fluoride, pH set with H_2SO_4). The fish are previously acclimated to the artificial softwater at pH = 6.5, Al = 0 ug/L for at least 2 weeks.

Our approach has been to "set" the water chemistry, using freshly prepared solutions, and then to move the water past the fish as quickly as possible to minimize pH shifts, speciation changes, and organic complexation reactions (1/2 replacement time ~20 min. in the experimental chambers). Note that the Al/pH combinations tested (plotted on Figure 3) are well below the solubility limits for amorphous aluminum hydroxide (and except in one case for gibbsite also), so supersaturated conditions are avoided, at least in the bulk water. The brook trout (Salvelinus fontinalis) has been studied as it is the salmonid endemic to the acid-threatened regions of North America, and the rainbow trout as it is a widely distributed reference species. For more detailed information on the brook trout studies, the reader is referred to Booth et al. (1988), Wood et al. (1988a,b,c), McDonald and Milligan (1988), and Walker et al. (1988), and on the rainbow trout studies to Goss and Wood (1988) and Playle et al. (1989).

Upon exposure to acidic pH alone in the absence of Al, brook trout exhibit transient and sublethal net losses of Na^+ and Cl^- across the gills in proportion to the severity of the pH (Figure 4); zero balance is re-established in 12 to 72 hours. The addition of Al to the exposures greatly exacerbates these losses and kills many of the fish within 24 to 72 hours (Figure 4). Terminal blood samples reflect these losses, with severely depressed ECF ions and dramatic hemoconcentration in lethal exposures, as previously seen in rainbow trout dying from pure acid stress. The cumulative ion losses prior to death in acid/Al exposures are not as large, however, suggesting that an additional toxic mechanism(s) may be involved (see below). In some cases, the brook trout do not die in the presence of Al, but rather return to zero or even positive balance and survive indefinitely.

Measurements of unidirectional fluxes across the gills with ^{22}Na demonstrate that initial losses reflect a combination of inhibited active uptake and stimulated diffusive efflux; the latter is quantitatively much more important. This constitutes an initial "shock" phase (0 - 12 hours) of heavy losses that may eventually kill the fish by inducing intolerable fluid volume and circulatory disturbances. The extent of cumulative ion loss during this period is a reliable predictor of eventual survival or death, even though that death may not occur for several more days. In trout that survive, there follows a "recovery" phase in which the efflux component is returned to control levels or below by 24 to 72 hours. Active influx remains depressed, however, for up to 10 days. The initial large efflux is thought to result from opening of the paracellular diffusion channels in the gills, and the later adaptive response from an effective closing of these channels, as discussed subsequently.

By 48 hours, the majority of the electrolyte loss that will occur has already done so. Cumulative 48-hour Na^+ loss and mortality are strongly correlated (Figure 4). These responses increase with Al concentration at any given acidic pH, and increase with pH at Al = 333 ug/L. Thus Al toxicity appears to be greatest at pH = 5.2, where total solubility is lowest and $Al(OH)^{2+}$ and $Al(OH)_2^+$ predominate (Figure 3).

The higher water calcium level (400 uequiv/L, still in the softwater range) generally reduces the extent of electrolyte loss at any particular pH/Al combination and removes the clear relationship between cumulative ion

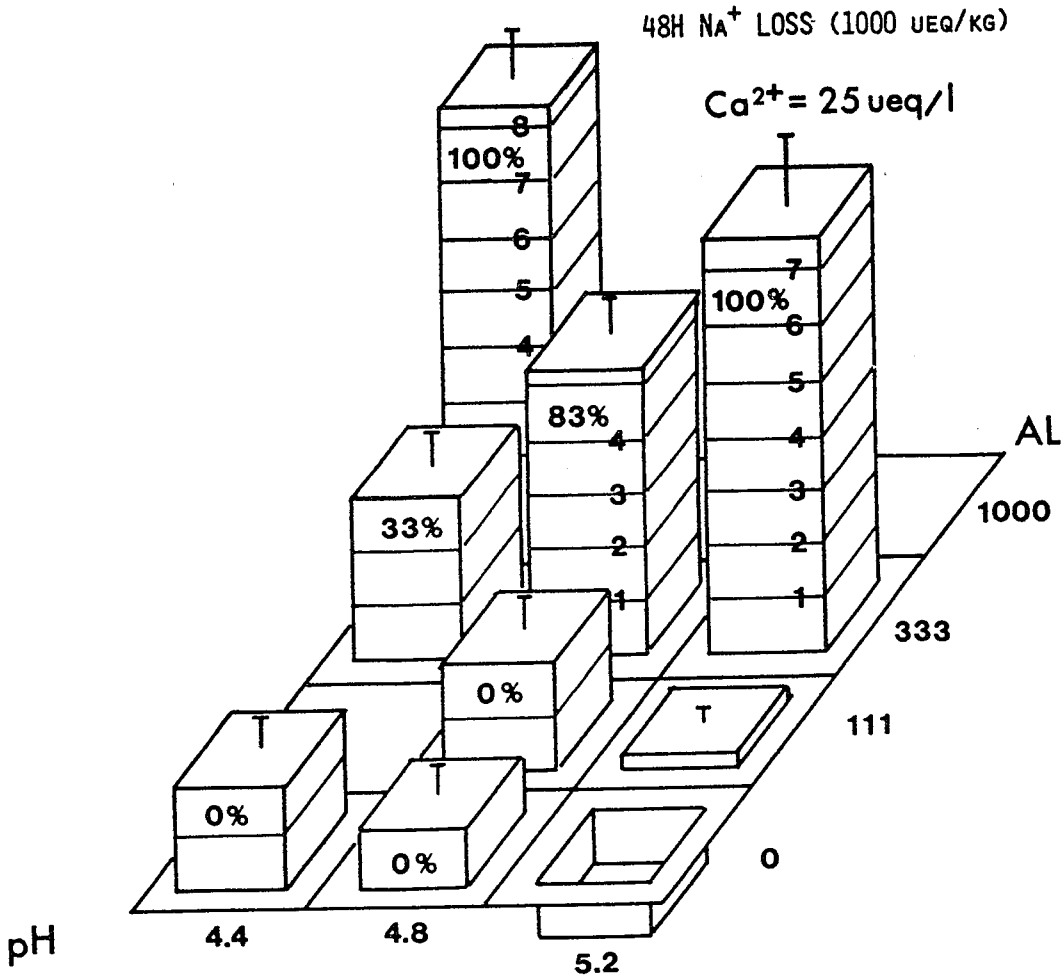

Figure 4. Cumulative net sodium losses of brook trout over the first 48 hours of exposure to various combinations of pH and aluminum in flowing softwater (Ca = 25 uequiv/L). Means ±1 SEM (n = 6 in each treatment). Percentage mortalities at the end of 10 days of exposure are indicated for each treatment. (modified from McDonald et al. 1988, Booth et al. 1988)

loss and mortality seen at lower calcium (25 uequiv/L; Figure 4). Nevertheless, a substantial number of fish still die, even though their ion losses and plasma Na^+ and Cl^- depressions are quite small. Under these conditions, respiratory failure, the second toxic mechanism alluded to previously, becomes the primary cause of death. This interference with O_2 uptake and CO_2 excretion across the gills is signalled by a pronounced hyperventilation accompanied by decreases in arterial blood O_2 tension (Pa_{O2}; Figure 5C) and O_2 content below typical venous values, and reciprocal increases in CO_2 tension and blood lactate. Although this phenomenon is most marked at the higher calcium level (Figure 5C) and higher pHs, it occurs prior to death in all treatments where Al is present (Figure 5B,E).

Although the responses of the rainbow trout are qualitatively similar to those of the brook trout, there are two important differences. Firstly, under any particular acid/Al condition, rainbows are far less tolerant, showing greater ion loss (Figure 6B), hemoconcentration (Figure 6C), and respiratory disturbance (Figure 6D), and earlier and larger percentage mortalities. These physiological results are in accord with field and toxicological data indicating that Salmo gairderi is the most sensitive, and Salvelinus fontinalis the most resistant, of a range of salmonids (Grande et al. 1978). Secondly, the contribution of respiratory disturbance to toxicity is greater in the rainbow trout under all conditions where Al is present, not just at the higher water calcium level. Indeed, unlike the brook trout, there is no evidence that respiratory disturbance is exacerbated by higher water calcium, although it is worsened by higher pH. These differences emphasize that generalization between species, even within the same Family, must proceed with caution.

BRANCHIAL MECHANISMS OF ACTION FOR ALUMINUM, ACID, AND CALCIUM

The preceding observations can be summarized by saying that acidity and aluminum both cause ionoregulatory toxicity, that calcium protects against ionoregulatory toxicity, that aluminum alone also causes respiratory toxicity, which may or may not be exacerbated by higher calcium, and that the extent of aluminum's ionoregulatory and respiratory effects appears to be greater at higher acidic pHs. Clearly the situation is complex, and any explanation(s) must take into account the chemistry of aluminum, the response(s) of the gill epithelium, and the nature of the interaction between the two. The final point is the critical one, about which we know very little at present. The ideas that follow are, therefore, highly speculative.

An important starting point is the observation that accumulation of aluminum on the gills (Figure 6A) appears to be directly associated with toxicity, and correlates well with physiological indices of disturbance (Figure 6B,C,D). Gill aluminum levels increase in a time- and concentration-dependent fashion, are greater at higher pH for any particular water Al level, are greatest in dying fish, and are clearly related to differences in species sensitivity (Figure 6A). We view this as a superficial uptake onto or into the gill cells, for neither we nor Neville (1985) can find any evidence of aluminum entry into the fish in exposures lasting up to 10 days.

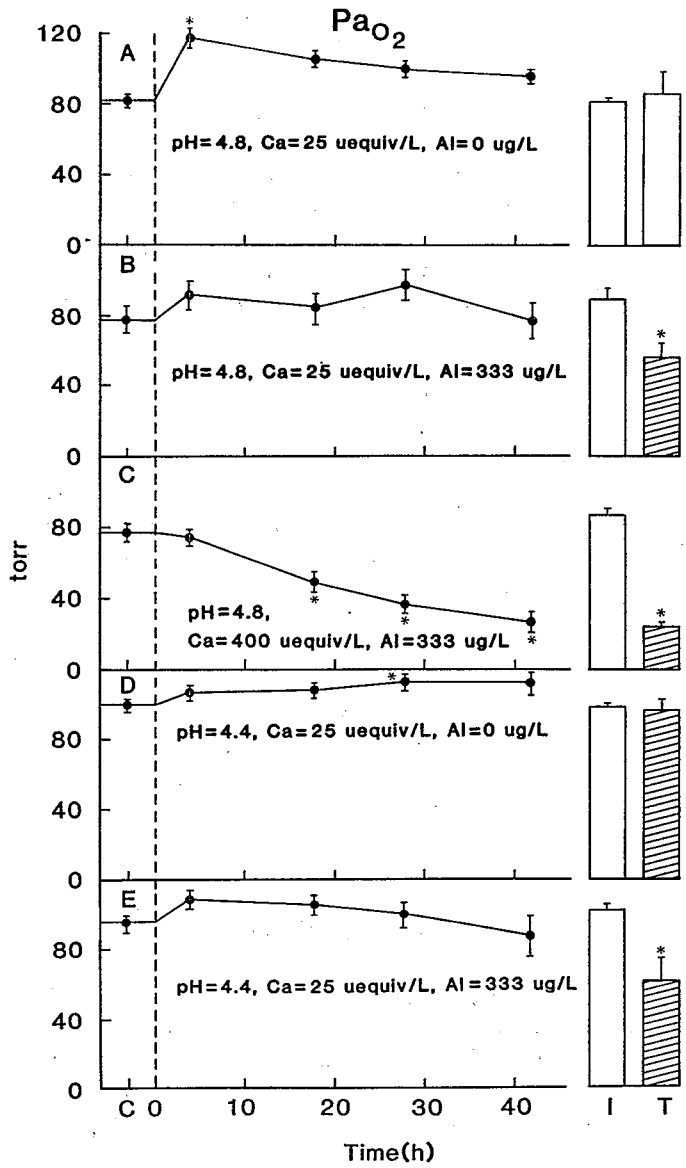

Figure 5. Changes in the O_2 tension of arterial blood (Pa_{O_2}) in chronically cannulated brook trout during 10 days exposure to five different pH/Ca/Al conditions in flowing softwater. Means ± SEM (n = 9 - 28 in each treatment). Data are shown from the control period until 48 hours for all fish that survived beyond this time in each exposure (i.e., the most resistant fish in each group). Additionally, terminal data (T, cross-hatched bars) representing the last measurements prior to death in fish dying at any time during the entire 10 days exposure are shown in bar graphs at right and compared with initial measurements (I, open bars) during the control period for these same fish. In panel A, none of the fish died, so data at 10 days exposure have been substituted for terminal data. Asterisks indicate significant differences ($p=<0.05$) from appropriate control or initial values. (from Wood et al. 1988a)

Why should aluminum accumulate in the first place? The branchial surface is rich in organic anions on mucus and cell surfaces, and these anions may avidly bind the cationic Al species that occur at low pH (Figure 3). Analysis of mucus fragments extruded by the fish or gently scraped from the gills of dead fish show very high aluminum contents. Furthermore, at bulk water pHs below about 5.5, the branchial micro-environment is much more alkaline than the inspired water (Figure 7A) probably due mainly to the efflux of NH_3 across the gills. As inflowing water encounters this more basic milieu, the formation of aluminum hydroxides will be favored (Figure 3), and perhaps more importantly, supersaturating conditions may occur, resulting in direct precipitation of polymers on the gills. The exponential relationship between pH and Al solubility is critical here (Figure 3).

Figure 7B illustrates that an aluminum concentration (111 ug/L) that would appear to be well below saturation at a bulk water pH of 4.8, is effectively right on the solubility limit, because the pH at the gills is raised to about 5.2 (Figure 7A). In turn, at a bulk water pH of 5.2, where this concentration appears to be at the solubility limit, it is in fact several-fold saturated (Figure 7B), because the pH is raised to about 5.5 at the gills (Figure 7A). This may explain why aluminum is more toxic at higher acidic pHs, although it is also possible that the less charged species are intrinsically more toxic; the two explanations are certainly not mutually exclusive.

Figure 8 presents a simple model of the branchial epithelium incorporating some of the ideas presented in this paper and recent histological work (Karlsson-Norrgren et al. 1986, Tietge et al. 1988, M.E. Mueller, pers. comm.). H^+ by itself (Figure 8B) inhibits the active Na^+ and Cl^- uptake processes through the chloride cells and, more importantly, increases their diffusive loss by opening up the tight junctions of the paracellular channels, perhaps by displacing bound Ca^{2+}; respiratory gas exchange is not affected. If aluminum also is present (Figure 8B), there is a precipitation of Al complexes and/or binding of Al to organic anions on the gill surface. In turn, this acts as an irritant that stimulates the production of additional mucus and induces an inflammatory response comprising edema, white cell invasion, lamellar clubbing and even fusion. A general thickening and distortion of the branchial epithelium results, which together with the mucus/Al layer, increases the transcellular diffusion distance from water to blood, thereby reducing O_2 and CO_2 exchange. At the same time, this distortion further opens the paracellular channels, increasing diffusive electrolyte loss, while the surface coating or Al cations themselves further inhibit active Na^+ and Cl^- uptake. Recovery, if it occurs, presumably reflects a reduction in the inflammatory response, a closing of the paracellular channels, and a reduction in the transcellular diffusion distance for O_2 and CO_2. Higher water calcium levels (Figure 8D) also help close the paracellular channels, thereby reducing ion efflux. In the brook trout, where calcium intensifies the gas exchange problem, we speculate that it acts in some way to also thicken the mucus/Al coat.

ACCLIMATION TO ACID/ALUMINUM STRESS

It was noted earlier that some individual trout may recover from an acid/Al challenge in the continued presence of the stressors, which raises the

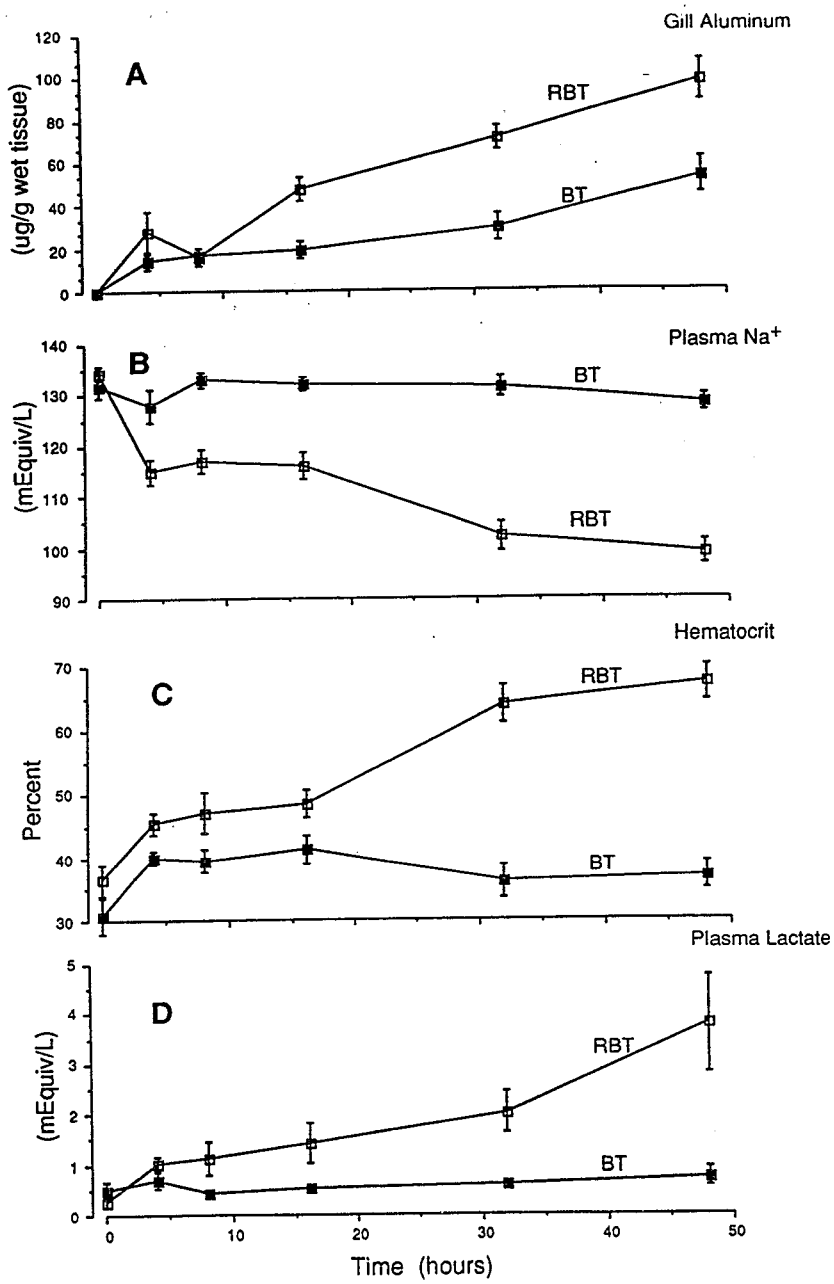

Figure 6. A comparison of the responses in (A) gill aluminum accumulation, (B) plasma sodium concentration, (C) hematocrit, and (D) blood lactate between juvenile brook trout (BT) and juvenile rainbow trout (RBT) exposed for 48 hours to pH = 4.8, Al = 111 ug/L, at Ca = 25 uequiv/L. Means ±1 SEM (n = 10 - 20 at each point). (D.G. McDonald and C.M. Wood, unpublished results)

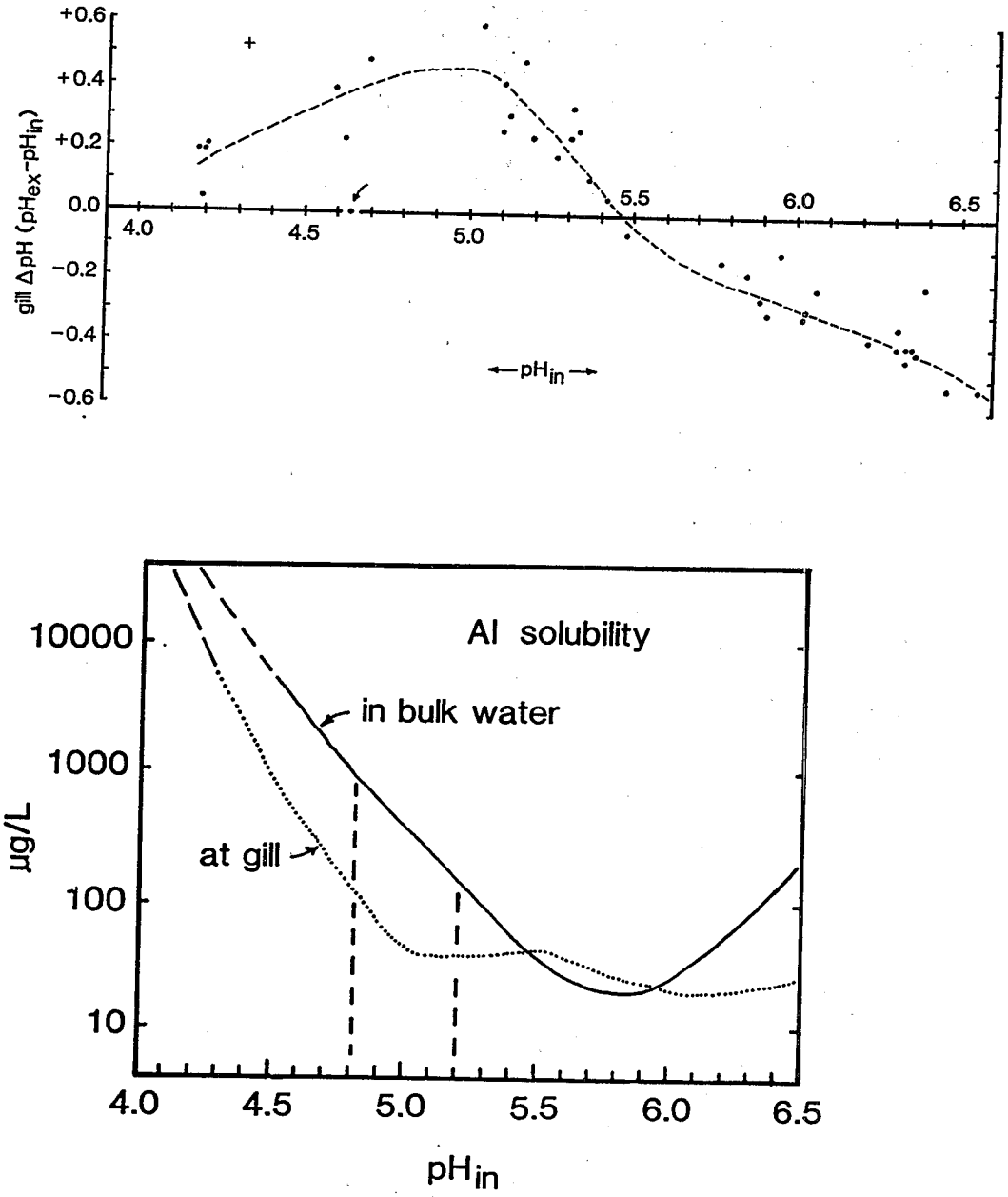

Figure 7. (A) The relationship between inspired bulk water pH and gill water pH in the rainbow trout in softwater. Each point represents an individual measurement. (B) The effect of this shift in water pH at the gills on the solubility of aluminum. See text for additional details. (R.C. Playle and C.M. Wood, unpublished results)

possibility that acclimation may occur. This is further suggested by field survey data (Schofield and Trojnar 1980, Kelso et al. 1986) from chronically acidified lakes, indicating that salmonids may survive in the field at aluminum levels known to be lethal in laboratory trials. Of course an alternate explanation is that of genetic differences and natural selection. Acclimation is defined as increased resistance to a more severe exposure acquired as a result of a previous sublethal exposure. Therefore, we have evaluated the physiological responses to long-term sublethal acid/Al exposures and subsequent more severe challenges. For more detailed information on these acclimation studies, the reader is referred to Wood et al. (1988b,c), McDonald and Milligan (1988), Audet et al. (1988), and Audet and Wood (1988).

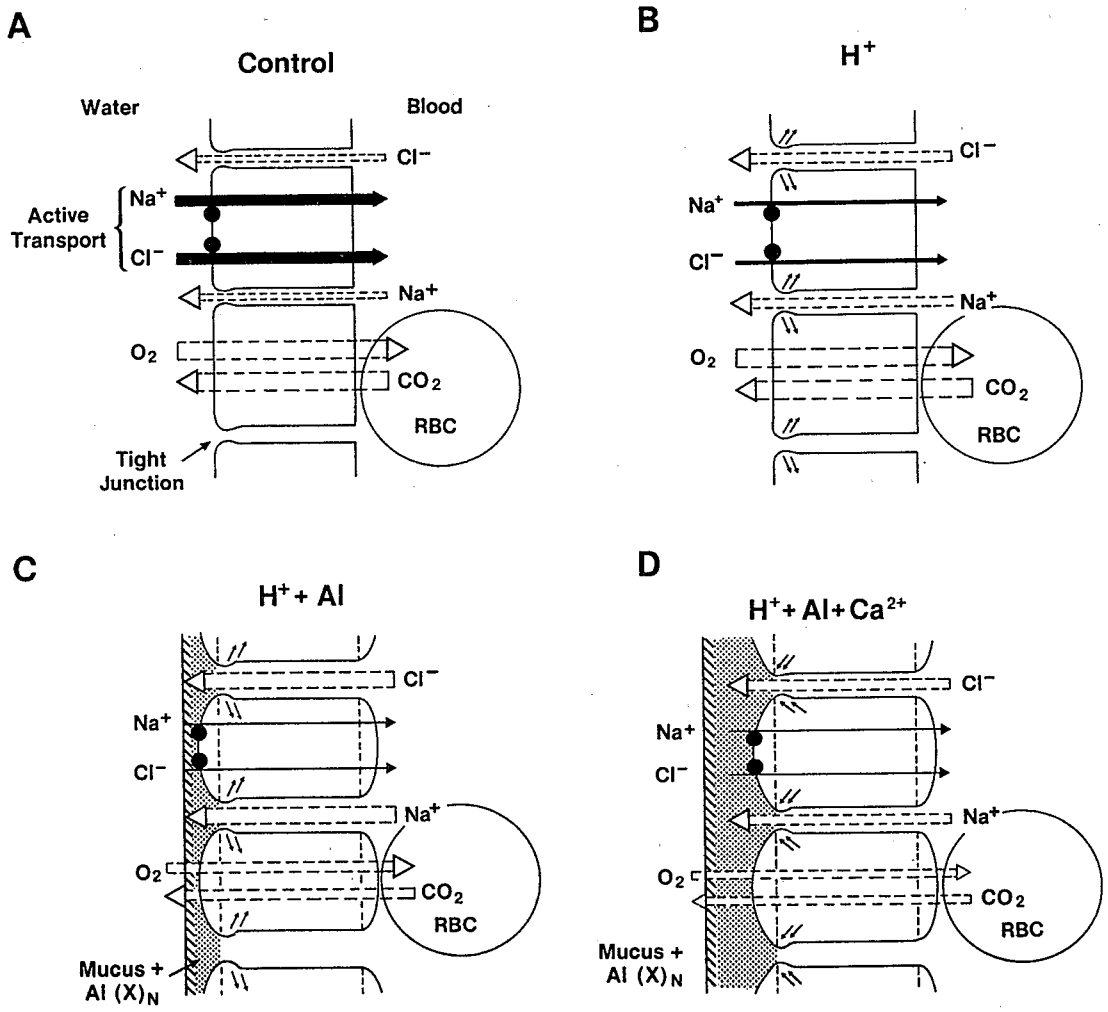

Figure 8. Hypothetical models of the actions of acid, aluminum, and calcium on ion and gas exchange across the gill epithelium of brook trout in softwater. (A) Control, (B) Acid alone present, (C) Acid plus aluminum present, and (D) Acid plus aluminum plus higher calcium levels present. Width of arrow indicates size of flux. (from Wood and McDonald 1987)

There is no evidence that salmonids have any ability to acclimate to pure acid. Rainbow trout exposed to sublethal low pH (4.8) in flowing softwater show a gradual loss of electrolytes and elevation of plasma glucose and cortisol levels, both of which are sensitive stress indicators. Although a plateau is reached after 4 to 7 weeks in most parameters, there is no recovery back to control levels. When these fish are challenged with a more severe exposure (pH = 4.0) they exhibit further ion losses and other physiological disturbances that are greater than those seen in previously unexposed trout. Thus sensitization, rather than acclimation, occurs.

The situation is very different for combined acid plus aluminum exposure. Brook trout held for 10 weeks in flowing softwater at pH = 5.2 plus 75 or 150 ug/L Al actually appear less stressed than those held in pH = 5.2 alone, based on plasma electrolytes, glucose, and cortisol, and rates of branchial sodium uptake. The mechanism is unknown but may be associated with a proliferation of branchial chloride and mucous cells seen in fish chronically exposed to low levels of aluminum (Tietge et al. 1988). When these fish are subjected to a more severe combined acid plus aluminum challenge (3 days at pH = 4.8, Al = 333 ug/L, which is lethal to the majority of previously unexposed trout), they exhibit 100% survival, and much smaller physiological disturbance. This acclimation appears to protect against both toxic mechanisms (ionoregulatory and respiratory distrubance), and is reflected in stress indicators (glucose, cortisol), respiratory parameters (ventilation, blood gases, lactate) and ionoregulatory parameters.

For example, Figure 9 illustrates that the cumulative Na^+ loss over the first 48 hours of challenge (pH = 4.8, Al = 333 ug/L) is approximately halved by pre-exposure to low levels of aluminum at pH = 5.2, and the depressions in plasma Na^+ and Cl^- correspondingly reduced or abolished. Note that the Na^+ loss is only reduced to the level that is caused by challenge with acid alone (pH = 4.8) and that pre-exposure to acid alone (pH = 5.2) provides no protection against acid plus aluminum challenge. In agreement with the rainbow trout experiments, this finding indicates that the acclimation is not to acid, but rather to aluminum.

What is/are the mechanism(s) of acclimation to aluminum? With other metals, acclimation has been traditionally associated with internal processes such as the induction of metal-binding proteins (e.g. metallothioneins) or the activation of excretion mechanisms. Aluminum appears to be entirely a surface-active toxicant, however, so these explanations appear unlikely.

As a first step in attacking this problem, we recently have followed a variety of physiological and histological parameters over time in juvenile brook trout exposed to sublethal aluminum. The goal was to see which parameters, if any, change as acclimation develops. After an initial sensitization, a significant and sustained increase in resistance (i.e. relative LT50) to more severe aluminum challenge occurs between days 10 and 13 of exposure to low levels of Al (75 or 150 ug/L) at pH = 5.2 (Figure 10A). Again exposure to acid alone (pH = 5.2) is relatively ineffective in inducing acclimation. Most internal parameters show little or no correlation with this time course, but there is a striking relationship between gill aluminum burden and the development of acclimation (Figure 10B). Despite continued low level exposure to

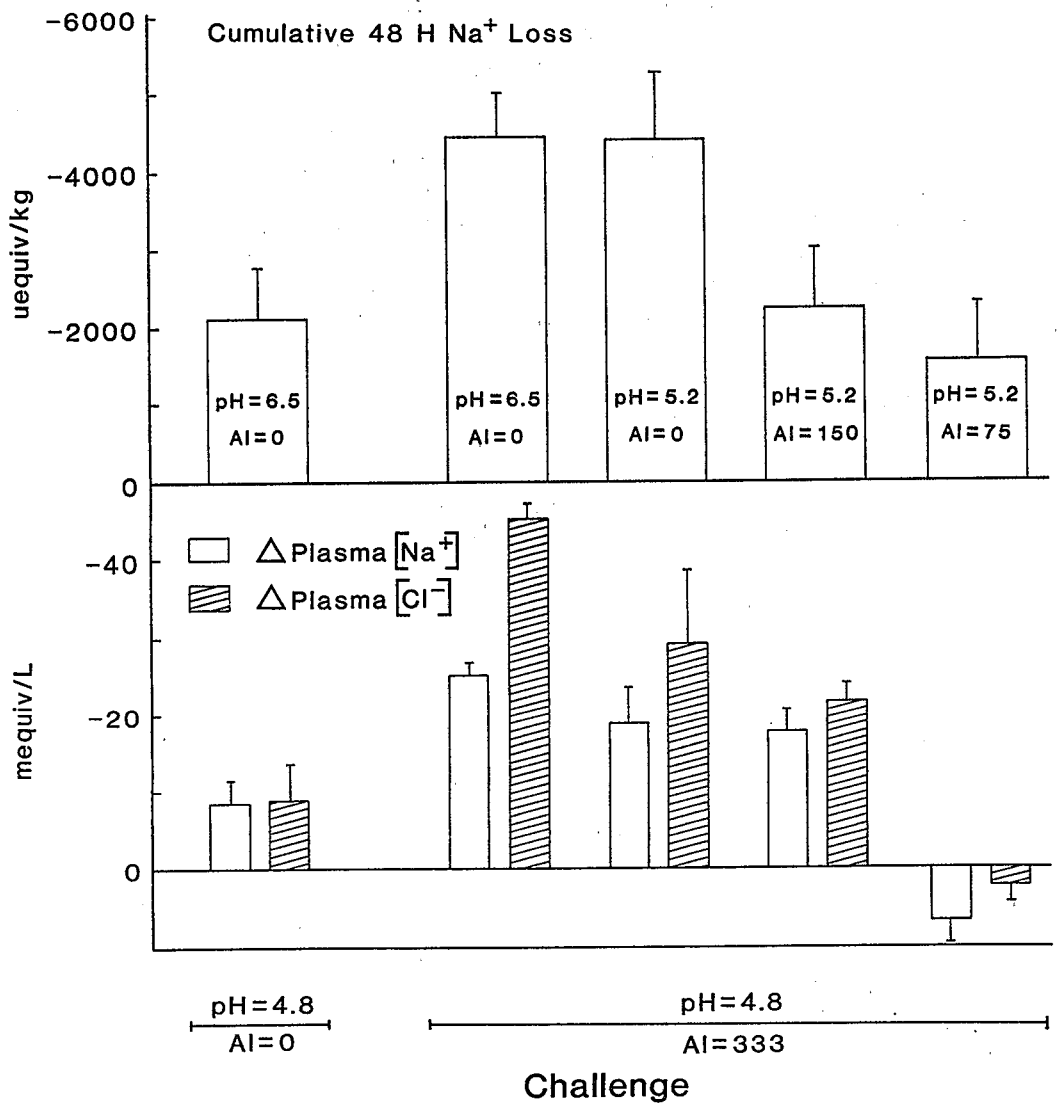

Figure 9. The effects of 10 weeks of sublethal exposure to conditions shown (in flowing softwater, Ca = 25 uequiv/L) on the responses of brook trout to subsequent challenge with a more severe low pH alone (4.8) or low pH plus Al (333 ug/L). (A) Cumulative net sodium losses over 48 hours of challenge. (B) Changes in plasma sodium and chloride concentrations over 48 hours of challenge. Means ±1 SEM (n = 6 in each group). (modified from Wood et al. 1988b)

the metal, the gill is in some way able to "clean" itself of bound or precipitated aluminum compounds.

A pronounced hypertrophy of branchial mucous cells occurs, so this may be associated with increased rates of mucus synthesis and sloughing as the cleansing mechanism. As the gill aluminum burden is reduced, so may be the inflammatory response, the paracellular permeability, and the diffusion distance for respiratory gases. Whatever the explanation, the nature of the gill surface appears to change so as to become less reactive to higher levels of waterborne aluminum during challenge.

CONCLUSION

The unifying theme of the research summarized here has been that the toxic actions of environmental acidity and associated aluminum stress, as well as the protective effects of calcium and the adaptive responses of recovery and acclimation, can all be interpreted at the level of gill epithelium. Nevertheless, our understanding of the chemical, physical, and morphological characteristics of the branchial surface remains rudimentary. A much finer characterization of this complex and delicate epithelium, together with its aqueous micro-environment, is required.

ACKNOWLEDGMENTS

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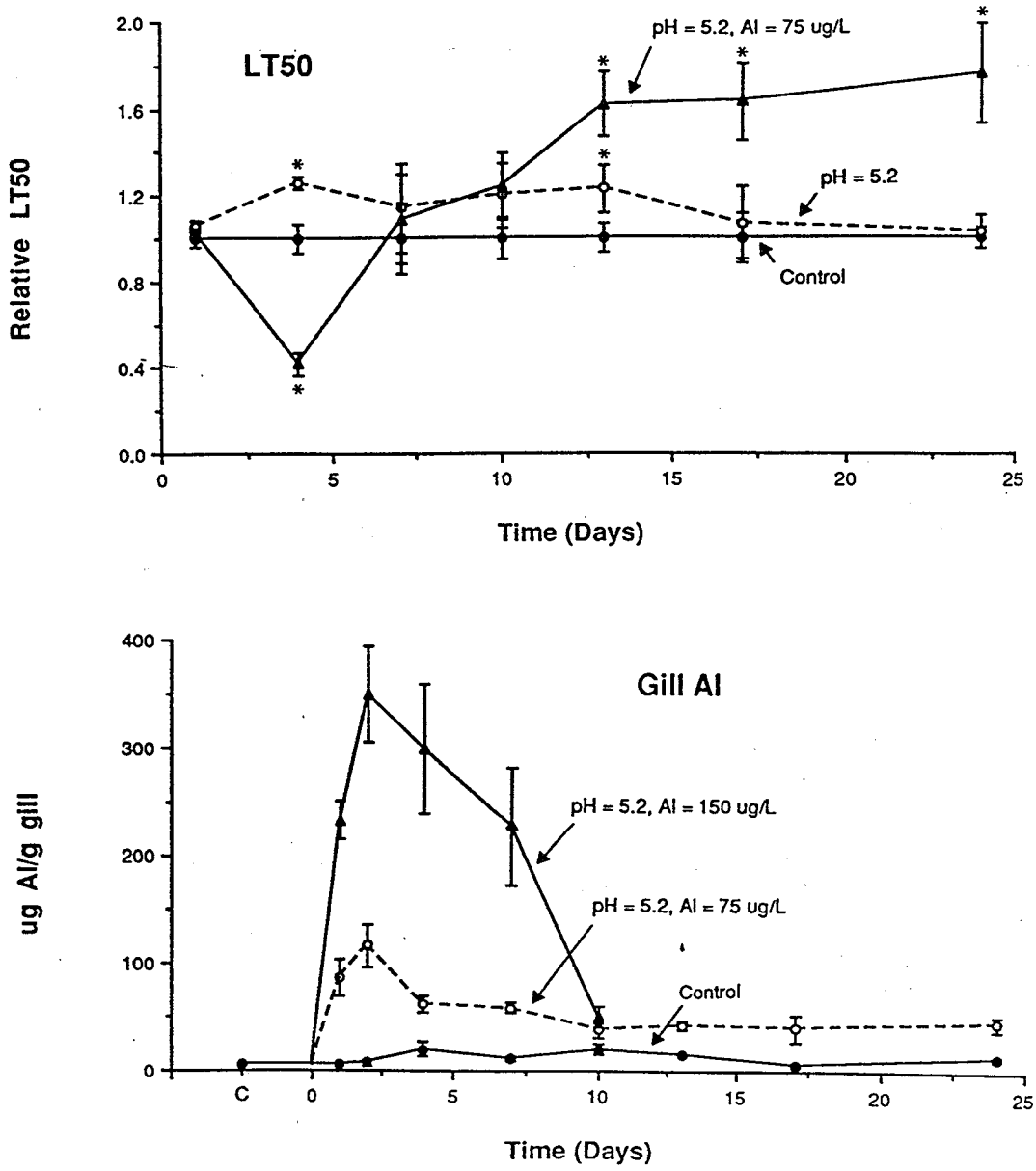

Figure 10. (A) The effects of sublethal exposure to pH = 6.5 alone (control), pH = 5.2 alone, and pH = 5.2 plus Al = 75 ug/L or 150 ug/L (in flowing softwater, Ca = 25 uequiv/L) on the mean lethal times (LT50, ±95% CL) of juvenile brook trout when challenged with Al = 1000 ug/L. The exposures started on Day 1. The LT50 data have been expressed as ratios to the control. Asterisks indicate means significantly different ($p<0.05$) from control. (B) Changes in gill aluminum content during these exposures. Means ±1 SEM (n = 10 at each point). (D.G. McDonald, C.H. Wood, R.G. Rhem, M.E. Mueller, D.R. Mount, and H.L. Bergman, unpublished results)

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AMMONIA TOXICITY TO FISHES

by
Robert V. Thurston[1]

INTRODUCTION

Ammonia is a naturally occurring product of metabolic degradation and, as a consequence, is commonly found in most aquatic environments. It also is frequently added to aquatic systems as a waste product from industrial, municipal, and agricultural processes. Ammonia is extremely toxic to fishes and other aquatic animals, and this toxicity is affected by several factors including water pH, dissolved oxygen content, temperature, concentration fluctuations, degree of salinity, presence of other chemicals, and prior acclimation. Short-term exposure of fishes to high concentrations of ammonia causes increased gill ventilation, hyperexcitability, loss of equilibrium, convulsions, and then death (Smart 1978, Thurston et al. 1981c). These effects are most likely the result of a direct effect of ammonia on the central nervous system. Chronic exposure of fishes to lesser concentrations of ammonia include damage to tissues, decreases in reproductive capacity (egg production, egg viability, spawning delay), decreases in growth, and increases in susceptibility to disease (Thurston et al. 1984, 1986). Chronic exposure also may cause progressive deterioration of other physiological functions, any one of which may be the ultimate cause of death (Randall and Wright 1987).

AQUEOUS AMMONIA EQUILIBRIUM

Ammonia assumes two chemical forms in aqueous solution, shown by the following equilibrium equation.

$$NH_3 + nH_2O = NH_3 \cdot nH_2O = NH_4^+ + OH^- + (n-1)H_2O.$$

These forms are the un-ionized ammonia species (NH_3), hydrogen-bonded to at least three ($n \geq 3$) water molecules (Butler 1964), and the ionized species (NH_4^+). The toxicity of ammonia is generally attributed to NH_3, which is an extremely soluble gas. Total ammonia is the sum of NH_3 and NH_4^+, and it is total ammonia that is most commonly measured in aqueous solutions; the concentration of NH_3 is then calculated. Tables of NH_3 concentration, as a function of total ammonia concentration, over a range of pH values from 5 to 12 and range of temperatures from 0 to 40 °C are available (Emerson et al. 1975, Thurston et al. 1979).

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The relative concentrations of ionized and un-ionized ammonia in a given solution are a function of the pH, temperature, and ionic strength of that solution. As pH increases, the concentration of NH_3 increases, while that of NH_4^+ decreases. For example, a pH increase from 7.0 to 8.0 in the temperature range of 0 to 30 °C results in a nearly 10-fold increase in the concentration of NH_3. Temperature increase also favors the NH_3 species, but to a lesser extent; a temperature increase of 5 degrees between 0 and 30 °C at pH 7.0 results in an NH_3 concentration increase of 40 to 50%. An increase in the ionic strength of a solution, at low concentrations, favors the NH_4^+ species. In natural waters with low to moderate amounts of dissolved solids (200-1000 mg/L) this effect will slightly reduce the concentration of NH_3, and the magnitude of this effect will vary with the composition of the water (Thurston et al. 1979).

RELATIONSHIP OF AMMONIA TO NITRITE AND NITRATE

Ammonia is interrelated with nitrite (NO_2^-) and nitrate (NO_3^-) through the process of nitrification, the biological oxidation of ammonia to nitrate. Under aerobic conditions ammonia is readily oxidized to nitrite by Nitrosomonas bacteria, and nitrite is then oxidized to nitrate by Nitrobacter bacteria. In well-oxygenated aquatic systems, the conversion of ammonia to nitrite is the rate-limiting step in the total process, and the conversion of nitrite to nitrate occurs fairly rapidly. Nitrite concentrations in most natural systems, therefore, are usually low, which is fortunate for aquatic life because nitrite, also, is extremely toxic to fishes. Nitrate, although generally present in all but the most oligotrophic surface waters, is relatively non-toxic to fishes. Where it is present in high concentrations, any problem is usually one of contributing to eutrophication rather than to toxicity.

ACUTE TOXICITY OF AMMONIA

There is some variation in the susceptibility of fishes to the ammonia toxicity. Representative 96-hour acute toxicity values (96-hour median lethal concentrations, or LC50 values) are listed in Table 1. From available data, salmonids appear to be among the most sensitive to acute ammonia exposure, and centrarchids, catfish, and some minnows the most tolerant. In addition to differences in susceptibility among fish species, there also can be differences in susceptibility at different life stages of a given species (Thurston and Russo 1983). Moreover, several factors can affect the toxicity of ammonia, including environmental pH, dissolved oxygen, temperature, salinity and ionic composition, previous acclimation and intermittency of exposure, and presence of other toxicants.

EFFECT OF pH

Some years ago researchers observed that the toxicity of ammonia solutions was greater at high pH values (Chipman 1934, Wuhrmann et al. 1947, Wuhrmann and Woker 1948), and because the percentage of NH_3 in total ammonia increases as solution pH increases, these authors concluded that NH_3 was the toxic form of ammonia in the water. They also concluded that NH_4^+ was non-toxic or appreciably less toxic. More recently, however, several researchers have reported that NH_3 is more toxic at low pH, separate from the effect of pH

Table 1. Representative acute toxicity values for un-ionized ammonia. (Values reported are ranges.)

Species	96-hour LC50 mg/L NH_3)	Reference
Pink salmon (*Oncorhynchus gorbuscha*)	0.08-0.1	Rice & Bailey (1980)
Mountain whitefish (*Prosopium williamsoni*)	0.14-0.47	Thurston & Meyn (1984)
Brown trout (*Salmo trutta*)	0.50-0.70	Thurston & Meyn (1984)
Rainbow trout (*Salmo gairdneri*)	0.16-1.1	Broderious & Smith (1979) Calamari et al. (1981) Thurston & Russo (1983)
Largemouth bass (*Micropterus salmoides*)	0.9-1.4	Roseboom & Richey (1977)
Smallmouth bass (*Micropterus dolomieui*)	0.69-1.8	Broderius et al. (1985)
Common carp (*Cyprinus carpio*)	2.2	Hasan & Macintosh (1986)
Red shiner (*Notropis lutrensis*)	2.8-3.2	Hazel et al. (1979)
Fathead minnow (*Pimephales promelas*)	0.75-3.4	Thurston et al. (1983)
Channel catfish (*Ictalurus punctatus*)	0.50-3.8	Colt & Tchobanoglous (1976) Roseboom & Richey (1977) Arthur et al. (1987)
Bluegill (*Lepomis macrochirus*)	0.55-3.0	Emery & Welch (1969) Roseboom & Richey (1977)

on the NH_3/NH_4^+ equilibrium in the water. This greater toxic effect has been demonstrated with prawn larvae and Daphnia, as well as with fishes (rainbow trout Salmo gairdneri, fathead minnow Pimephales promelas, coho salmon Onchorhynchus kisutch, chinook salmon O. tshawytscha, channel catfish Ictalurus punctatus, and green sunfish Lepomis cyanellus) (Tabata 1962, Sousa et al. 1974, Robinson-Wilson and Seim 1975, Armstrong et al. 1978, Hillaby and Randall 1979, Tomasso et al. 1980, Thurston et al. 1981c, McCormick et al. 1984, Broderius et al. 1985, Sheehan and Lewis 1986). Results of one such study (Thurston et al. 1981c) showed that there was a decrease in the 96-hour LC50 value for NH_3 for rainbow trout (Figure 1) and fathead minnows as pH decreased, indicating that fishes were more susceptible to NH_3 at low pH. In longer exposures, Broderius et al. (1985) also reported a marked effect of pH on the toxicity of NH_3 in a study on smallmouth bass (Micropterus dolomieui) from late embryo through 32 days of exposure. The 32-day "no-observed-effect"

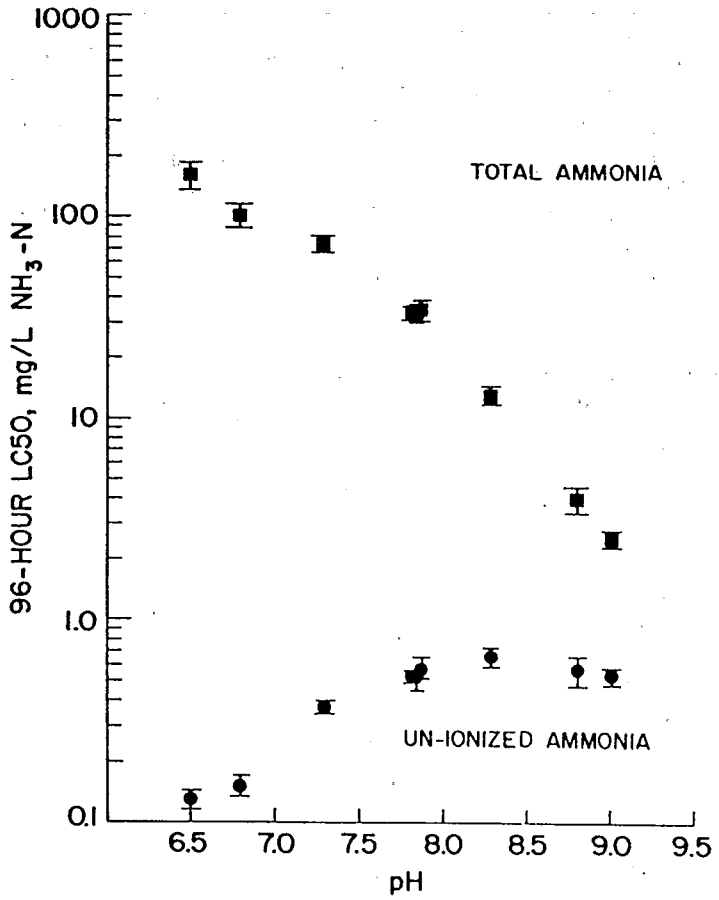

Figure 1. Acute toxicityof ammonia (96-hour LC50 values) to rainbow trout at various environmental pH values. Error bars are 95% confidence intervals. (Reprinted with permission from Thurston et al. 1981c; copyright 1981, American Chemical Society)

concentrations radically decreased from 0.61 to 0.044 mg/L NH_3 as pH was lowered from 8.68 to 6.60. In a preliminary study, we have observed that the toxicity of NH_3 also increased at pH values above 9 (Figure 2) (Russo et al. 1988).

EFFECT OF DISSOLVED OXYGEN

Several researchers, working with a variety of fish species, have reported that the toxicity of ammonia increases when dissolved oxygen concentrations decrease (Wuhrmann 1952, Wuhrmann and Woker 1953, Downing and Merkens 1955, Merkens and Downing 1957, Alabaster et al. 1979, Thurston et al. 1981b). Discharges of ammonia frequently are associated with reduced oxygen concentrations in the receiving water, so the effect of dissolved oxygen on ammonia toxicity can be important.

EFFECT OF TEMPERATURE

Many researchers have reported that ammonia is less toxic to fishes at temperatures near the higher end of their normal environmental range than near the lower end, but not all studies support this. Colt and Tchobanoglous (1976) reported a decrease in acute toxicity of ammonia to channel catfish with an increase in temperature over the range 22 to 30 °C, and Roseboom and Richey (1977) reported that bluegill (Lepomis macrochirus), largemouth bass (Micropterus salmoides), and channel catfish were more susceptible to ammonia toxicity at 22 °C than at 28 to 30 °C. Arthur et al. (1987) conducted 96-hour ammonia toxicity tests on white sucker (Catostomus commersoni), walleye (Stizostedion vitreum vitreum), rainbow trout, fathead minnow, and channel catfish at temperatures ranging from 3.4 to 26 °C. Except for channel catfish, none of the tests showed a progressive increase in LC50 with increasing water temperature, and no clear relationship was found between ammonia toxicity and temperature; the 96-hour LC50 values for channel catfish were 0.50, 0.98, and 1.29 mg/L NH_3 at 3.5, 14.6, and 19.6 °C.

FLUCTUATING CONCENTRATIONS AND ACCLIMATION

Fish are frequently subjected to fluctuating concentrations of ammonia as a consequence of diurnal changes. Also, episodic "slugs" of ammonia, as a result of accidental spills or intentional discharges into rivers and lakes, are common. Thurston et al. (1981a) studied the acutely toxic effects of fluctuating concentrations of ammonia on rainbow trout and cutthroat trout (Salmo clarki) and reported that fish were able to withstand short-term excursions slightly above acutely toxic concentrations without any apparent long-term adverse effects, provided the high ammonia concentrations were followed by periods of low ammonia concentration. However, they also reported that fish were better able to withstand steady concentrations of ammonia, over 96 hours, than they were fluctuating concentrations having mean values comparable to those of the steady concentration for the same length of time. Increased resistance to acute toxicity from ammonia as a consequence of prior exposure to low ammonia concentrations has been reported by Vamos (1963), Malacea (1968), Schulze-Wiehenbrauck (1976), Redner and Stickney (1979), Thurston et al. (1981a), and Alabaster et al. (1983). Soderberg and co-

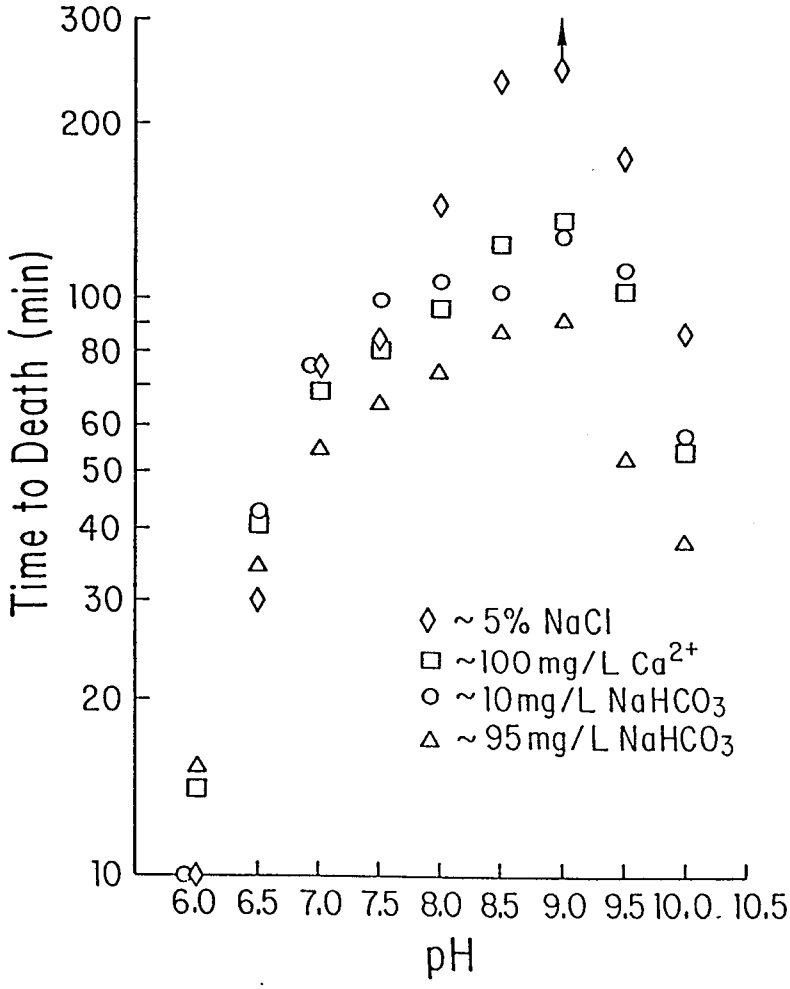

Figure 2. Effect of pH and different water chemistry variables on the acute toxicity of un-ioninized ammonia to coho salmon alevins. (Reprinted with permission from Russo et al. 1988; American Fisheries Society)

workers (1983), however, have reported that tissue damage in rainbow trout reared for 4 months in earthen ponds was correlated with ammonia concentration extremes, as opposed to ammonia averages.

MIXTURES OF AMMONIA AND OTHER CHEMICALS

Information on the toxic effects of ammonia as a result of exposure in combination with other chemicals is limited, but an increase in ionic strength appears to reduce ammonia toxicity, separate from the effect of ionic activity on the NH_3/NH_4^+ equilibrium. Increased calcium concentrations in the water have been reported to decrease ammonia toxicity to channel catfish (Tomasso et al. 1980), and an increase in water salinity has been reported to reduce the toxicity of ammonia to Atlantic salmon (Salmo salar) (Alabaster et al. 1979).

A decrease in the toxicity of ammonia to juvenile coho salmon with the addition of sodium chloride to the test solution also has been observed in our laboratories (Figure 2).

Wuhrmann and Woker (1948) and Broderius and Smith (1979) studied combinations of ammonia and hydrogen cyanide and reported synergistic effects of the two toxicants. The toxicity of ammonia and phenol combined was reported to approximate the toxicity of either ammonia or phenol when tested separately (Herbert 1962). Research has also been conducted on the effect of ammonia in combination with copper (Herbert and Vandyke 1964), zinc (Ministry of Technology, U.K. 1962), and combinations of these (Brown et al. 1969); the toxicity of ammonia in combination with these other toxicants appears to be simply additive.

CHRONIC TOXICITY OF AMMONIA

The longest ammonia exposure study reported in the literature is a life-cycle test on rainbow trout, conducted over a 5-year period (Thurston et al. 1984). Fish were tested at five exposure concentrations over the range 0.01 to 0.07 mg/L NH_3. Parental fish were exposed for 11 months, the first filial generation (F_1) for 4 years, and the second filial generation (F_2) for 5 months. The parental fish spawned of their own volition, and F_1 fish were manually spawned at 4 years of age. Blood ammonia concentrations in F_1 fish were positively correlated with ammonia concentrations in the test waters, and histopathological lesions were common in parental and F_1 fish at 0.02 mg/L NH_3 and higher. No other major effects were observed. Viable eggs were produced by both generations at all ammonia concentrations tested, and there was no significant correlation between ammonia concentration and numbers of egg lots spawned, total numbers of eggs produced, numbers of viable eggs, growth of progeny, or mortality of parents or progeny in any of the generations tested.

Effects of ammonia on fish reproduction and survival were observed, however, in two life-cycle tests on fathead minnows (Thurston et al. 1986). Fish were tested at five exposure concentrations for 1 year over the range of 0.07 to 0.96 mg/L NH_3. Growth and survival of, and egg production by, parental fish were all affected at 0.96 mg/L NH_3; no effects were observed on growth or survival of parental fish at 0.44 mg/L, or on egg production or viability at 0.37 mg/L. Growth and survival of F_1 larvae were not affected at 0.36 mg/L NH_3 (the highest concentration at which they were tested), and egg hatching success was not affected at 0.19 mg/L, but was at 0.37 mg/L. Brain lesions were common in parental fish at all stages of their development at exposure concentrations of 0.21 mg/L NH_3 and higher, but not at 0.11 mg/L. In summary, the chronic effects threshold concentration for these tests on fathead minnows, based on survival, growth and reproductive success, was estimated to be 0.27 mg/L NH_3; based on histological damage, it was estimated to be only half that, 0.15 mg/L NH_3.

Other studies at sublethal concentrations of ammonia, lasting from 1 week to 3 months, have been reported for both salmonid and non-salmonid species (Department of the Environment, U.K. 1972, Robinette 1976, Schulze-Wiehenbrauck 1976, Burkhalter and Kaya 1977, Thurston et al. 1978, Rice and Bailey 1980). At exposure concentrations as low as 0.002 to 0.15 mg/L NH_3, fish

showed reduced food uptake and assimilation, accompanied by growth inhibition. In the range of 0.04 to 0.4 mg/L NH_3, effects reported for a variety of fish species, under a wide range of water test conditions, included leucopenia and diminished numbers of red blood cells, inflammation and degeneration of gills and kidneys, and lowered resistance to disease (Reichenbach-Klinke 1967, Flis 1968, Smart 1976, Thurston et al. 1978, Peters et al. 1984, Soderberg 1985, Dabrowska and Wlasow 1986, Lang et al. 1987).

Carline et al. (1987) tested the effects of domestic wastewater on the survival, growth, swimming performance, and gill tissue of brown trout (Salmo trutta) for 12 months at concentrations from 0.005 to 0.066 mg/L NH_3. No significant effects were reported for survival, growth, or swimming performance, but degree of damage to the gills was directly related to effluent concentrations. Mitchell and Cech (1983) have implicated chlorine as confounding ammonia-caused gill damage. Burrows (1964) tested rainbow trout for 6 weeks at concentrations as low as 0.003 mg/L NH_3 (recalculated from the author's original data) and reported extensive gill damage, but Daoust and Ferguson (1984) tested that same species at concentrations up to 0.4 mg/L NH_3 for 90 days and did not observe "characteristic" gill damage. Meade and Herman (1986) reported that lake trout (Salvelinus namaycush), reared for 8 weeks in a water re-use system, showed gill and kidney damage of the kind reported to be caused by ammonia, and yet the ammonia concentrations to which these fish were exposed averaged 0.001 mg/L NH_3 or less within each 2-week period, and the highest recorded value was 0.003 mg/L. Their conclusion was that "parameters other than ammonia concentrations are significant factors in branchial irritation" of fish under culture.

In an extensive review article, Meade (1985) states as part of his summary: "The accumulating evidence indicates that gill hyperplasia, reported as characteristic of ammonia poisoning, is probably not caused by un-ionized ammonia. ... All end products of metabolism probably have not been identified, and certainly their interactions in water of various qualities and in various fish culture systems have not been determined."

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ENVIRONMENTAL TOLERANCE OF SOME MARINE FISH: APPLICATIONS IN MARICULTURE MANAGEMENT

by

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INTRODUCTION

Most studies of environmental effects on marine fish have confined themselves to evaluating physiological responses of a few species. Few studies compare the environmental tolerances of different species. Moreover, differences in experimental protocols make it difficult to compare tolerances and responses between studies (Davis 1975).

Although it has long been recognized that the success or failure of mariculture operations may hinge on the choice of species (Avault 1986), in the absence of scientific data, the suitability of a species for a particular hydrographic condition in a culture site can only be determined by experience or by trial and error. Hydrographic conditions, however, vary and fluctuate greatly both spatially and temporally. As a result, knowledge of environmental tolerances and requirements of marine fish provides a valuable scientific basis for selecting appropriate species to suit different culture conditions and is of obvious importance from the viewpoint of mariculture management and development.

The tolerance of a species to a particular environmental factor may be assessed by mortality and the behavioral and physiological responses of the fish. In this context, the time for 50% of the experimental population to show mortality (LT_{50} value), and the time for 50% of the experimental population to exhibit abnormal behavioral changes (BC_{50} value) can serve as useful tolerance indicators with which the relative tolerance of different species in relation to a particular environmental factor can be assessed and compared. This paper presents experimental data on oxygen, salinity, and temperature tolerances of some common mariculture fish in the Southeast Asia regions, and the results are related to fish kill statistics collected for 10 years in Hong Kong. The application value of these tolerance data in mariculture management also are discussed.

MATERIALS AND METHODS

All fish used for the experiment had been cultured in sea cages (salinity, 30 to 32%; dissolved oxygen, 5 to 8 mg O_2 L^{-1}; water temperature,

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25 to 28°C) for about a year. Experimental fish weighing in the range of 150 to 250 g were selected and acclimated in the laboratory (salinity, 30‰; dissolved oxygen, 8 to 8.5 mg O_2 L^{-1}; temperature, 25 ± 1°C) for at least 48 hours prior to the experiment and were not fed throughout the entire acclimation and experimental period.

OXYGEN TOLERANCE

A continuous flow system that provided a constant, controlled level of ambient oxygen was set up for the hypoxic tolerance experiment (for detailed design of the system, see Wu and Woo 1984). Twenty individuals of each of the nine species listed in Table 1 were acclimated in the experimental system for 24 hours prior to experiments, during which period normoxic water (25 ± 1°C) was provided. The levels of oxygen were adjusted to 4, 2.5, 1.0 and 0.5 mg O_2 L^{-1} within 15 minutes and the fish were exposed to the desired dissolved oxygen level for 7 hours. The time at which any fish began to show any stress symptom (i.e. loss of balance, abnormal swimming pattern, jerking) was noted and the time of death of any fish was recorded.

TABLE 1. TIME (minutes) FOR 50% OF VARIOUS EXPERIMENTAL FISH TO SHOW MORTALITY (LT_{50}) AND ABNORMAL BEHAVIOR (BC_{50})[a,b]

Species	0.5 mg O_2 l^{-1}		1.0 mg O_2 l^{-1}	
	BC_{50}	LT_{50}	BC_{50}	LT_{50}
Chrysophrys major	TQC	TQC	20	60
Rhabdosarga sarba	TQC	TQC	130	225
Siganus oramin	TQC	7	10	177
Lutjanus ruselli	TQC	14	78	(30%)
Epinephelus akaara	70	92	110	160
Mylio macrocephalus	30	100	NBC	NM
Epinephelus awoara	150	200	190	270
Lates calcarifer	373	393	NBC	NM
Epinephelus tauvina	(10%)	(10%)	NBC	NM

[a]Actual mortalities are shown in parenthesis for mortality-behavior changes occurring in less than 50% of the population throughout the experimental period.

[b]TQC, too quick to count; NBC, no behavioral changes; NM, no mortality.

To test whether there is any correlation between hypoxic tolerance and metabolic rate of different species, the standard oxygen consumption rate of each species at 25°C (ambient oxygen level) was measured. Five of each species were placed individually in sealed Plexiglas chambers (20 x 12 x 12 cm) and were placed in experimental tanks of the continuous flow systems. Fish were allowed to adapt to the experimental condition for 24 hours before measurement. Water was allowed to flow into the chamber through the inlets and siphoned out from the outlet of the chambers. The flow rate was adjusted so that there was a measurable difference of about 0.5 mg O_2 L^{-1} between the inflow and outflow of the sealed chambers. An empty chamber identical with that containing the animal was set up as a control, and the oxygen uptake of the fish was calculated using the following equation.

$$q = (X_0 - X_1)\ (dV/dt)_1 - (X_0 - X_2)(dV/dt)_2$$

where q is the oxygen uptake, X_0 is the oxygen concentration at the inflow, X_1 is the oxygen concentration at the outflow of the experimental chamber, X_2 is the oxygen concentration at the outflow of the chamber, $(dV/dt)_1$ is the flow rate of the experimental chamber, and $(dV/dt)_2$ is the flow rate of the control chamber. The oxygen consumption rate then is calculated and expressed in terms of mg O_2 g body wet wt.$^{-1}$ min.$^{-1}$

SALINITY TOLERANCE

After acclimation, twenty individuals of each of the 13 species listed in Table 3 were transferred abruptly to 3, 5 and 10% sea water (temperature = 25 ± 1°C) for 14 days in the laboratory. In addition, twenty individuals of S. oramin E. akaara, and m. macrocephalus were transferred to freshwater (0%). The time at which any fish began to show any stress symptom was noted and the time of death of any fish was recorded.

TEMPERATURE TOLERANCE

A continuous flow system in which a constant water temperature of 25 ± 0.2°C was set up for the temperature tolerance experiment. Twenty individuals of each of the eight species listed in Table 4 were acclimated in the system for 24 hours prior to experiments. The temperature of the system was gradually brought to 12 ± 0.2°C within 1 hour, and the fish then were subjected to the experimental temperature for 7 hours. The time at which any fish began to show any stress symptom was noted and the time of death of any fish was recorded.

FISH KILL STATISTICS

Since 1976, all major fish kills in Hong Kong have been reported to the Agriculture and Fisheries Department. In most of these cases, investigations were carried out to ascertain the cause of the kill and to assess the total loss as well as the species affected. Over 40% of the major kills recorded was attributable to oxygen depletions resulting from red tides and algal

blooms; the total loss between 1976 and 1988 was estimated at 124 tonnes, valued at approximately US $1 million.

RESULTS

OXYGEN TOLERANCE

No observable stress symptom can be found for all species at 2.5 mg O_2 L^{-1} and above. Most of the species turned into a noticeable lighter color when oxygen dropped below 2.5 mg O_2 L^{-1}, and this phenomenon was particularly marked for the Sparidae. Time required for 50% of each species to show stress symptoms and mortality are shown in Table 1. Large variations were found in hypoxic tolerance amongst the nine species tested. For example, 50% of C. major exhibited abnormal symptoms within 20 minutes at 1 mg O_2 L^{-1}, whereas L. calcarifer and E. tauvina did not show any abnormal symptoms within the 7-hour exposure. Similarly, all C. major and R. sarga died before the oxygen level dropped to 0.5 mg O_2 L^{-1}, whereas only 10% of E. tauvina showed abnormal symptoms and mortality under the same condition throughout the 7-hour experimental period.

Oxygen consumption rates for the nine species tested are shown in Table 2. The metabolic rate of intolerant species (e.g. C. major and R. sarba) was more than two times higher than those of tolerant species (e.g. E. tauvina and

TABLE 2. OXYGEN CONSUMPTION RATE OF VARIOUS SPECIES AT 25°C AND NORMOXIA (Species arranged in ascending order according to their LT_{50} values at 0.5 mg O_2 l^{-1}).

Species	Oxygen consumption rate, (μg O_2 g^{-1} $min.^{-1}$
Chrysophrys major	4.65 ± 0.81
Rabdosarga sarba	4.88 ± 0.33
Siganus oramin	9.73 ± 1.42
Lutjanus ruselli	5.93 ± 0.21
Epinephelus akaara	3.48 ± 0.12
Mylio macrocephalus	2.84 ± 0.39
Epinephelus awoara	2.20 ± 0.40
Lates calcarifer	1.98 ± 0.14
Epinephelus tauvina	1.99 ± 0.53

L. calcarifer). A Spearman rank correlation test showed a significant correlation between the rank orders of the LT_{50} and BC_{50} values at both 1.0 and 0.5 mg O_2 L^{-1} (r=0.986 and 0.790, respectively). A significant negative correlation was found between oxygen consumption rate and LT_{50} and BC_{50} values of the species tested at 0.5 mg O_2 L^{-1} (r= -0.732 and -0.673, respectively).

SALINITY TOLERANCE

LT_{50} values for each species at different salinities are shown in Table 3. Except for Caranx equula, no mortalities were found for any species at the end of 14 days of exposure to 30 and 10%. No mortality was found in 6 species exposed to 5%. Three species survived without any abnormal behavior in 3%, and 10% of M. macrocephalus survived without any abnormal behavior at the end of the 14 days of exposure to freshwater.

TEMPERATURE TOLERANCE

Except for S. oramin, which exhibited a 20% mortality, no mortality was found for the other 7 species in a 7-hour exposure to 12°C. Time for 50% of experimental animals to exhibit abnormal behavior is shown in Table 4. S. oramin and Lates calcarifer showed a very quick behavioral response to the lowering of water temperature, followed in order by E. tauvina. R. sarba and C. major. No behavioral changes were found for E. akaara, E. aworara, and M. macrocephalus during a 7-hour exposure to 12°C.

FISH KILL STATISTICS

Species involved in fish kills due to oxygen depletion in Hong Kong from 1976 to 1986 are shown in Table 5. C. major and R. sarba were involved in 17 and 11 incidents out of 19 major fish kills, respectively. L. calcarifer and E. tauvina were never reported in any fish kills caused by oxygen depletion although they also are commonly cultured. Table 6 shows the stock and percentage killed of each species in a fish kill in which a dissolved oxygen level of 0.8 mg O_2 L^{-1} was recorded. In this incident, only C. major, R. sarba, L. ruselli, and E. akaara were killed. M. macrocephalus and E. tauvina were not affected although they also were cultured at the same site.

Statistics of fish kills caused by cold spells (with water temperature <15°C for more than 7 days) in Hong Kong are shown in Table 7. L. calcarifer, E. tauvina were found to be involved in all the five recorded incidents. E. akaara, E. awoara, and M. macrocephalus were never reported although these species also were cultured.

DISCUSSION

Most warm water fish survive under a wider range of environmental conditions than do cold water species (Parker and Davis 1981). In this study, the species tested showed large variations in their tolerance to hypoxia, temperature, and salinity, and several species showed remarkable tolerance to

TABLE 3. LT_{50} VALUES (hours) OF VARIOUS MARINE FISH KEPT UNDER DIFFERENT HYPO-OSMOTIC SALINITIES FOR 14 DAYS (n=20).[a,b]

Species	Salinity 0%	3%	5%	10%
Siganus oramin	1.2	2.3	NM	NM
Caranx equula	-	3.1	42.5	126.0
Parapristipoma trilineatum	-	4.4	38.0	NM
Chrysophrys major	-	4.8	11.1	NM
Lethrinus nebulosus	-	9.1	86.0	NM
Epinephelus akaara	2.5	31.3	NM	NM
Plotosus anguillaris	-	40.0	55.0	NM
Pomadasys hasta	-	70.0	(30%)	NM
Epinephelus awoara	-	76.0	NM	NM
Rhabdosarga sarba	-	96.0	(10%)	NM
Therapon jarbua	-	NM	NM	NM
Lutjanua russelli	-	NM	NM	NM
Mylio macrocephalus	60.0	NM	NM	NM

[a]Actual mortalities shown in parentheses for mortalities of less than 50% within the experimental period (Wu and Woo 1982).

[b]NM, no mortality.

one or more parameters. For example, the black sea bream, Mylio macrocephalus, was very tolerant to all three environmental parameters tested.

Results of hypoxic tolerance experiments show that Lates calcarifer, Epinephelus tauvina and Mylio macrocephalus are tolerant of, whereas Chrysophrys major and Rhabdosarga sarba are particularly susceptible to, hypoxic conditions. Hypoxic tolerance is negatively related to the metabolic rate of the species. It appears that species that have a lower metabolic rate survive better in a hypoxic environment because less oxygen would be required to sustain essential biological functions. The hypoxic tolerance of fish species derived from laboratory results agrees closely with these established from analyses of fish kill statistics. C. major and R. sarba always suffer

TABLE 4. TIME (minutes) FOR 50% OF EXPERIMENTAL ANIMALS TO EXHIBIT ABNORMAL BEHAVIOR (BC_{50}) AT C (n=20)

Species	BC_{50}	Mortality, %
Siganus oramin	TQC	20
Lates calcarifer	1	-
Epinephelus tauvina	20	-
Rhabdosarga sarba	50	-
Chrysorphyrs major	89	-
Epinephelus akaara	NBC	-
Epinephelus awoara	NBC	-
Mylio macrocephalus	NBC	-

[a]NBC, no behavioral changes; TQC, too quick to count

heavy loss in fish kills caused by oxygen depletions; other species normally are affected to a much lesser extent.

Conversely, L. calcarifer and E. tauvina, which have never been reported in any fish kills due to oxygen depletion, were found to be highly tolerant to hypoxia in the laboratory experiments. Detailed biochemical studies on Ephinephelus akaara and Mylio macrocephalus exposed to hypoxic conditions by Woo and Wu (1984) revealed no increases in serum and tissue lactate and only slight changes in other tissue metabolites and electrolytes at 4.0 to 2.5 mg O_2 L^{-1} for these two species, indicating that they can obtain enough oxygen to prevent anaerobiosis in this regime. Marked elevations of serum lactate and serum Na^+, K^+ and Ca^{2+} were found and osmoregulation failure occurred, however, when oxygen values fell below 1 mg O_2 L^{-1}.

The overall results suggest, therefore, that hypoxic tolerant species such as Lates calcarifer and M. macrocephalus may be cultured in eutrophic waters in which oxygen depletion is more likely to occur and that culturing sensitive species such as C. major and R. sarba should be avoided in the same environment. Nevertheless, the quick, abnormal behavioral response to hypoxia exhibited by C. major and R. sarba may serve as an effective indicator with which culturists could detect the onset of oxygen depletion at an early stage and aerate their cages to prevent loss of their stock. Such a "biological indicator" system has been proven to be successful in Hong Kong.

TABLE 5. NUMBER OF INCIDENTS IN WHICH A PARTICULAR SPECIES WAS INVOLVED IN 19 MAJOR FISH KILLS CAUSED BY OXYGEN DEPLETION IN HONG KONG, 1976 to 1987

Species	No. of incidents involving species in 19 fish kills
Chrysophrys major	17
Rhabosarga sarba	11
Epinephelus akaara	6
Seriola purpurascens	5
Lutjanus ruselli	4
Mylio macrocephalus	2
Mylio latus	2
Lutjanus sanguinensis	2

Results of the salinity tolerance experiments show that 10 out of the 13 species tested are euryhaline and survive without any abnormal behavior and tissue hydration for more than 2 weeks in salinities above 10% (Wu and Woo 1983), suggesting that such salinities are normally not an important limiting factor for culturing most of these species. Further experiments carried out on E. akaara and M. macrocephalus showed only transient disturbance of various electrolytes and metabolites in the case of the latter at salinities above 12%, suggesting that physiological disturbance is unlikely to occur in salinity above this regime (Woo and Wu 1982). Dendrinos and Thorpe (1985) also found that the bass (Dicentrarchus labrax) survives in salinities between 5 and 33% for over 12 months, and food and protein conversion efficiencies were maximal at 25 and 30%. It may be hypothesized further that, because less energy would be required for osmoregulation in an iso-osmotic environment, energy saved might be channelled to tissue production. The possibility of increasing mariculture productivity by culturing eurhaline species in an environment of reduced salinity (e.g. estuary) should be explored.

Results from temperature tolerance experiments indicated that S. oramin, L. calcarifer, and E. tauvina are relatively sensitive to cold temperature, whereas E. akaara, e. awoara and M. macrocephalus are more tolerant. Again, the laboratory findings were clearly supported by fish kill statistics in Hong Kong. High mortality of the former three species were found during cold spells when water temperatures fell below 15°C for a prolonged period. Conversely, Mylio macrocephalus, Epinephelus akaara, and Epinephelus awoara, which showed no abnormal behavior at 12°C in the laboratory, have never been

reported upon in fish kills caused by cold spells. The results, therefore, indicate over-wintering problems for L. calcarifer and E. tauvina in Hong Kong. Shortening of the grown-out period by, for example, importing larger fingerlings might minimize the risk of fish kills for these two species in a severe winter.

Menasveta (1981) found that the range of upper lethal temperature for 24 species of marine fish in the Gulf of Thailand is fairly narrow (34°C to 37.5°C). The upper lethal temperatures of Chrysophrys major and Mylio macrocephalus were found to be 32 and 36°C, respectively (Woo and Fung 1980, Woo per. com). The low tolerance of C. major to high water temperature suggests that this species is less suitable for culturing in shallow waters (i.e. < 3m, the shallowest thermocline in the coastal waters of Hong Kong) where water may easily be heated by solar radiation in the summer to a temperature beyond the lethal limit of this species.

TABLE 6. INVOLVEMENT OF VARIOUS SPECIES IN FISH KILLS AT PO TOI O HONG KONG IN WHICH A DISSOLVED OXYGEN LEVEL OF 0.8 mg O_2 l^{-1} WAS RECORDED

Species	Stock (t)	Loss (t)	Loss (%)
Chrysophrys major	8.1	0.1	1.2
Rhabdosarga sarba	20.3	0.8	3.9
Lutjanus russelli	2.8	0.1	3.6
Epinephelus akaara	0.8	0.1	12.5
Sub-total	32.0	1.1	3.4
Epinephleus awoara	3.0	0	0
Epinephleus tauvina	2.4	0	0
Mylio latus	1.3	0	0
Mylio macrocephalus	9.0	0	0
Lutjanus argentimaculatus	1.2	0	0
Others	2.1	0	0
Sub-total	22.5	0	0

TABLE 7. NUMBER OF INCIDENTS IN WHICH A PARTICULAR SPECIES WAS INVOLVED IN FIVE MAJOR FISH KILLS CAUSED BY COLD SPELLS (water temperature less than 15°C for more than 7 days) IN HONG KONG, 1982 to 1987

Species	No. of incidents involving species in five fish kills
Lates calcarifer	5
Epinephelus tauvina	5
Lutjanus sanguinensis	4
Rhabdosarga sarba	1

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STUDIES ON PHYSIOLOGY OF PHOTOTAXIS OF FISH AND MARINE ANIMALS IN CHINA

by

Daren He[1]

INTRODUCTION

Lightfishing has a long history in China. It can be traced back to ancient times when fishermen used pine torches to lure cuttle fish and squid in the sea and used lamps to lure crabs and torches as an aid to catching cormorants. As early as 1930s, the fishermen of Dongshan, Fujian, Nanao, and Guangdong used gaslamps to lure and catch round scads and sardines. With the depletion of demersal fish resources, many countries in the world have began to exploit the rich resources of pelagic fish. At present, lightfishing is an important means of catching pelagic fish. With the development of science and technology and the improvements in lighting technology, lightfishing has become an advanced means of production that plays an important role in marine fishery in many countries. China's coastal waters abound in varieties and quantities of pelagic fish, of which chub mackerel, round scads, horse mackerel, round herring, sardines, etc. have already become the major objects for purse seine fishing with light.

In the early 1960s, we carried out successful experiments in underwater lamp fishing with the fishermen of Xiamen, Dongshan, and other areas. From the late 1960s to the early 1970s, the fishermen of Xiamen, Dongshan, etc. made tremendous strides in seine fishing using lights installed on motorized junks. In the early 1970s, Shanghai Fishery Company and other units began to develop purse seine fishing with lights, furthering the exploitation of the marine pelagic fish resource of our country and raising the proportion of the pelaghic fish yield in the total. To raise the light fishing industry further, we must solve the problems that arise in production. Several questions are involved. What is the optimum color light source for round scad and chub mackerel? What is their optimum illumination? Why is the gathering effect of light under moonlight not so ideal? Why is the phototaxis so bad during the breeding season?

With these questions in mind, we, together with our colleagues in the Shanghai Institute of Physiology and the East China Sea Fisheries Research

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With these questions in mind, we, together with our colleagues in the Shanghai Institute of Physiology and the East China Sea Fisheries Research Institute, made comprehensive studies of the phototactic character of the main marine pelagic fish such as round scads and chub mackerel using ethological, electro-physiological and visual-pigment-biochemical methods in the early 1970s. We also tested new light sources such as thallium-indium lamp on the sea and achieved excellent results. Our achievements won the China National Science Conference Prize in 1978. Later we continued with our studies on the theory and application of the mechanism of phototaxis of fish and marine animals, vision physiology, and ethology. This paper gives a summary of the study in this field in China.

He Daren, Yu Wentsao, and others have studied the phototactic behaviour of round scad (Decapterus maruadsi), chub mackerel (Pneumatophorus japonicus), sardine (Sardinella perforata), silverside (Atherina bleekeri), needlefish (Ablennes anastomella), cardinal fish (Apogon lineatus), Mugil carinatus and squid (Loliog duvaucelii) using the photogradient method; Zheng Meili, He Daren, and others have studied the behavioural physiology of cuttlefish (Sepiella maindroni); Luo Huiming and others have studied that of young eel (Anguilla japonica) and swimming crab (Portunus trituberculatus). Their studies included threshold of reaction to light, optimum illumination, reaction to colored light, and the influence of background light on phototactic behaviour. The exposition of the above-mentioned characteristics of animals can provide a scientific basis for the selection of the optimum artificial light source in production, the determination of effective attraction range, and the calculation of gathering rate.

There were three major findings. First, young fish and adults of round scad and chub mackerel showed positive phototaxis under the horizontal photogradient of between 10^{-1} and 10^{3}|x. Second, the phototaxis of young fish was stronger than that of adults. Third, the optimum illumination of young chub mackerel was 10^{-2} to 14 lx in water temperature of 24.5 to 27°C. In both single and group experiments, the results of response of chub mackerel are basically the same, and the response of chub mackerel are basically the same, and the response for the group experiment is even more stable.

We also did comparison experiments on needlefish, cardinal fish and Mugil carinatus, the results are: (1) needlefish were always distributed in the relatively stronger illumination region of each series of photogradient; (2) under the same conditions, cardinal fish were constantly distributed in the relatively weaker illumination region of each series of photogradient; (3) the phototaxis of the young fish and adults of round scad and chub mackerel and the young of Mugil carinatus remained between the former two. Chub mackerel and round scad both belong to the type of fish that show positive reaction to light but do not tend to the strongest light. When a comparison was made between these two, round scad showed stronger phototaxis than chub mackerel. Therefore, both fish can be the objects of light fishing. The optimum illumination regions of squid and cuttlefish were 10^{-1} to 10^{1}lx and 10^{-2} to 10^{1} lx, respectively. Both belong to the type that tends to weak light. The optimum illumination of swimming crab was 10^{-3} to 10^{-2}lx. With growth development, the optimum illumination of young eels changed from 10^{-1} to 10^{1}lx

to 10^{-2} to 10^{-1}lx. This shows that the character of phototaxis differs in different stages of development.

Some fish and marine animals can show selective response and color vision to colored light. Study in this field is of great importance to light source color selection in production. Under dark adaptation, the phototactic rate of young and adult round scad is highest to blue-green light while that to red light is the lowest. After the transition from dark to light adaptation, the phototactic rate to yellow-green light is the highest. Chub mackerel show the highest phototactic rate to violet and red lights under both dark and light adaptations. The peak value of its spectral sensitivity remains to blue and green lights. The author points out that the optimum light color is different from the sensitivity light color. The squid shows the greatest phototactic rate to blue light.

Moonlight, functioning as background light, poses a practical problem in production. Our tests showed that background light invariably reduced the phototaxis of the above-mentioned animals; the influence of background light could be restrained by increasing the light intensity of illumination; the background light had a stronger influence on the animals of weaker phototaxis than on those of stronger phototaxis. Within the range of 10^1 to 10^3lx, however, squids showed a higher phototactic rate with background light than without it. Such an unusual phenomenon was associated with the sexual maturity and moonlight night reproduction of squids. Therefore, contrary to the common phototactic fish, the yield of squids can be raised if we fish with a hook and a line at moonlight night during their breeding season with properly increased light source intensity. Water temperature can influence the phototaxis of young round scads and increase their phototaxis within a relatively lower optimum temperature range.

He Daren and others (1983) also have studied the feeding intensity of juvenile mullets (Mugil sp.) under different illumination and probed into the role of vision during a fish's feeding. The results show that the illumination intensity has a very strong influence on the feeding intensity of juvenile mullet to daphnia. Its feeding intensity reaches the maximum under the illumination of 10^2lx; the feeding rate reaches the highest under 10^2lx and reaches the maximum within 20 minutes.

The peak of feeding activity of young mullet appears only under certain illumination conditions and is closely related to the feeding activity under natural conditions and circadian rhythm. Our tests have confirmed the important role of vision in their feeding activity and provided a basis for determining the environmental brightness and bait quantity at the time of throwing in the bait.

Since 1984, He Daren and others have studied the optomotor reaction of fish. They studied the optomotor reaction characters of the young fish of gray mullet (Mugil cephalus), perch (Lateolabrax japonicus), porgy (Sparus latus), carp (Cyprinus carpio), grass carp (Ctenopharyngodon idellus), etc. Liu Lidong and others (1986) also have made an overall study on the optomotor reaction of Nile tilapia and its influencing factors. Their study included the influences of environmental illumination, the screen speed of rotation,

water temperature, structure of visual field, body length, etc., on the optomotor reaction of tilapia. They also described the characters of reaction of individual, population, and monoculars. The young fish of this kind has typical optomotor reaction under the illumination of 10^{-5} to 10^{4}lx. Within a certain range, the optomotor reaction of fish increases with rising environmental illumination and water temperature and declines with increasing screen rotation speed and body length. The fish reacts most effectively to black-and-white vertical stripes, less effectively to oblique stripes, and none to horizontal stripes. The reaction of optomotor reaction intensifies with the increasing width and number of vertical stripes within a certain range. There is no obvious difference in the reaction of individuals and populations. The reaction of monocular fish is evidently weaker than that of normal-binocular ones, and the former has an obvious orientation. He Daren and Zhou Shijie have studied the sensibility of young prawn (Penaeus orientalis) to moving objects and its relationship with environmental illumination. They also have studied the optomotor reaction of young porgy under conditions of colored light.

ELECTROPHYSIOLOGICAL STUDIES

For the past dozen years and more, much has been done in the field of electrophysiology of fish and in marine animal vision. As the phototactic character of fish is related directly to its vision function, the determination of the electro-activity of different levels of peripheral and central neurons is one of the ideal indexes in the study of physiology of phototaxis. The main work in China is to study the ERG character with rough electrodes. After an overall study on the ERG of round scad and chub mackerel, Young Xiongli and others have found that their ERG had the character of the retina of mixed type and had obvious off-response and that its b-wave was highly sensitive to the oxygen deficiency: Background light could noticeably raise the threshold of retina sensitivity. Zheng Weiyun and others have found through their study on red grouper (Epinephelus akaara) that its retina has a two photoreceptor system of rod and cone, but they have not found any typical character of mixed retina in the ERG. They believe that the cone of such fish has degenerated and is adapted to weaklight vision without the ability of color discrimination.

Yang Xiongli and others (1978) have determined the spectral sensitivity of the ERG b-waves of round scads and chub mackerel and found that the peaks of spectral sensitivity curves of the two fishes under dark vision are 490 and 480nm, respectively. The peaks of spectral sensitivity curves under light vision shift to 520 and 525 nm, respectively. They also have studied the induction potential of the tectum of chub mackerel. With the latent period of induced potential as the index, the spectral sensitivity curves thus obtained basically correspond with those under ERG dark vision. Therefore, it is believed that the spectral sensitivity remains unchanged in the visual center. Zheng Weiyun, Chai Minjuan, Chen Zhong, Liu Lidong, and others have studied the ERG character of cuttle fish, swimming crab and prawn (Penaeus penillatus) and showed that the ERGs of these invertebrates are cornea negative wave one positive off-response. This is identical with the structure and arrangement of their retina. Through analyzing the ERG spectral sensitivity of mud crab

(Scylla serrsta) under different adaptation, Yang Xiongli and others have suggested that its compound eye has only a single receptive system and revealed the circadian rhythms of its ERG sensibility. They also have drawn a schematic diagram for control of circadian rhythms in the ERG of the crab. Chai Minjuan and others have made some comparative studies on the ERG circadian rhythms of several kinds of crustaceans (mud crab, tiger head crab Orithyia mammillaris, swimming crab and prawn). In recent years, scientists of our country also have begun to study micro-electrodes. Yang Xiongli and others have recorded the horizontal cell potential of retina on the body of fresh water fish.

STUDIES ON BIOCHEMISTRY OF VISUAL PIGMENT AND HISTOLOGY OF RETINA

As visual pigment forms the material basis of photoreception of retina, great importance has been attached to its study. Not much work has been done in the field of biochemistry of visual pigment; however, Chen Ming and others have determined the rod visual pigment of round scad, chub mackerel, black carp (Mylopharyngodon piceus) grass carp, silver carp (Hypophthalmichthys malitrix), bighead (Aristichthys hobilis), tilapia, and others. Black carp, grass carp, silver carp and bighead have pigment of simple retinene2; the peak values of their spectral absorption are 530, 528, 525 and 527 nm, respectively. Chub mackerel show the pigment of retinenel and the peak value of its absorption is 500nm. Round scad contain two kinds of rhodopsin and their peak values of absorption are 488 and 510nm, respectively. Tilapia contains the mixed pigment of retinenel and retinene2 and their peak values of absorption are 500 and 522 nm, respectively.

Zheng Meili and others (1980) have studied the histological structure of the retina of cuttlefish. Xu Yonggan and others (1986) have made histology-physiological studies on the retina of gray mullet, porgy, red grouper, round scad and sardine (Sardinella auritus). They also have studied submicroscopic structure and probed into the relationship between the rètina morphology and the function of the five fishes and the ecological environment. Xu Yonggan, He Daren, and Zhang Houquan have made further studies on the retinomotor reaction of the young fishes of porgy and gray mullet.

The paper has so far given a brief account of the research work that has been done in China for the past dozen years or more in the fields of the phototactic physiology of fish and other marine animals. There is still much room for further study. For example, the varieties of fish that have been studied are still just a few. Round scads and chub mackerel have been studied through various methods, but most of this fishes have been studied only in some particular aspects. Few studies have been made on the biochemistry of visual pigment; a systematic study on the factors that influence the phototaxis of fish is still lacking. It also is necessary to probe deeply into the mechanism of phototaxis. As regards test methods, in the past, more work was done in the laboratory than actual observation on the sea. Experimental techniques must be improved--for example, automatic record-analysis equipment should take the place of naked-eye observation in the study of ethology and micro-electrodes and ultramicroelectrodes should be used in electrophysiology. All these must be improved in future work.

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IDENTIFIED SEX PHEROMONES IN FISH: ENDOCRINE AND BEHAVIORAL EFFECTS

by

Norman E. Stacey[1] and Peter W. Sorensen[2]

Teleost fish use pheromones for a variety of functions--identifying individuals, kin groups, and species; attracting conspecifics (i.e., schooling); triggering alarm or fright reactions that signal the injury of a conspecific; and synchronizing reproductive activities both between and within the sexes (see reviews by Colombò et al. 1982; Lambert et al. 1986; Liley 1982; Liley and Stacey 1983; Stacey et al. 1986, 1987). In nearly all cases, the evidence for such pheromonal activity comes from studies in which individuals are exposed to an unpurified conspecific odor (holding water or tissue preparation). Although such an experimental approach may convincingly demonstrate pheromonal activity, until a pheromone is identified chemically and available for testing, it is impossible to conduct carefully controlled experiments to investigate the mechanism of pheromone action.

In the past few years, we have been fortunate in identifying several sex pheromones released by female goldfish that trigger physiological and behavioral responses in the male (Sorensen and Stacey, in press). The fact that these pheromones are commercially available sex hormones has greatly facilitated our studies. More importantly, our findings raise the possibility that fish commonly use sex hormones and their metabolites as sex pheromones; if true, both fundamental and applied aspects of fish sex pheromone research could expand rapidly in the near future. Indeed, there now seems no doubt that sex pheromones soon could be applied effectively to the problems of controlled reproduction of cultured fishes.

FEMALE SEX PHEROMONES IN GOLDFISH

Ovarian growth and vitellogenesis in goldfish occur during fall and winter (Stacey 1987). In the spring, increasing water temperature and aquatic vegetation (spawning substrate) trigger ovulation in females with post-vitellogenic ovaries. Females typically spawn only a few times during the late spring spawning season. As is typical of many cyprinids, goldfish are

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group- or gang-spawners, many males following or chasing an ovulated female so as to be close when she releases her oocytes in floating vegetation. It is highly likely that male goldfish have evolved adaptive responses to the odors of periovulatory females as a result of selective pressures exerted by two key aspects of goldfish reproduction--the existence of interacting mixed-sex groups prior to spawning and the intense male competition for females during spawning. Because many cultured cyprinids display reproductive activities comparable to those of goldfish, they also may have evolved similar sex pheromone systems.

The sex pheromones we have identified in female goldfish are released during the brief periovulatory period that commences with the start of the preovulatory gonadotropin (GtH) surge and ends with the completion of oviposition approximately 15 hours later. This ovulatory process is closely synchronized with the light:dark cycle, the GtH surge commencing in mid-photophase, ovulation (follicular rupture) occurring in the latter half of scotophase, and oviposition extending over several hours during early morning (Stacey et al. 1979). Between the onset of the GtH surge and ovulation, females release a preovulatory pheromone with "primer" effects on the male reproductive endocrine system (Stacey et al. in press). Later, between ovulation and the completion of oviposition, females release a postovulatory pheromone with "releaser" effects on male sexual behavior (Sorensen et al. 1988). A schematic model of the sequential function of these two pheromones is presented in Figure 1.

PREOVULATORY PRIMER PHEROMONE

Evidence of a preovulatory primer pheromone in goldfish was first provided by Kobayashi et al. (1986a) who showed that, in males placed in contact with preovulatory females, blood GtH rises in synchrony with the female's GtH surge. Because the odor of preovulatory females was sufficient to trigger a GtH increase in males and because cutting the olfactory tracts blocked the response, Kobayashi et al. (1986b) suggested that the GtH increase in males was triggered by a female sex pheromone. Our studies have confirmed these findings and demonstrated that the preovulatory pheromone is 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-P), the ovarian steroid that induces oocyte final maturation (migration and breakdown of the nucleus; Nagahama et al. 1983). We have shown that preovulatory females release 17,20β-P to the water where it then functions as a pheromone to rapidly increase circulating GtH levels of males (Stacey and Sorensen 1986, Dulka et al. 1987, Stacey et al. in press). This increase in male GtH then stimulates an increase in sperm production. Males exposed to the preovulatory female pheromone thus benefit from increased stores of releasable sperm (and therefore increased fertility) by the time the female ovulates and begins to spawn.

Blood levels of 17,20β-P begin to increase several hours after the start of the preovulatory GtH surge, reach peak levels 4 hours prior to ovulation, and return to basal levels near the time of ovulation (Kobayashi et al. 1987, Stacey et al. in press); peak levels are at least 30 times those preceding and following the periovulatory surge. Because the periovulatory profile of 17,20β-P released to the water is essentially the same as that in the blood

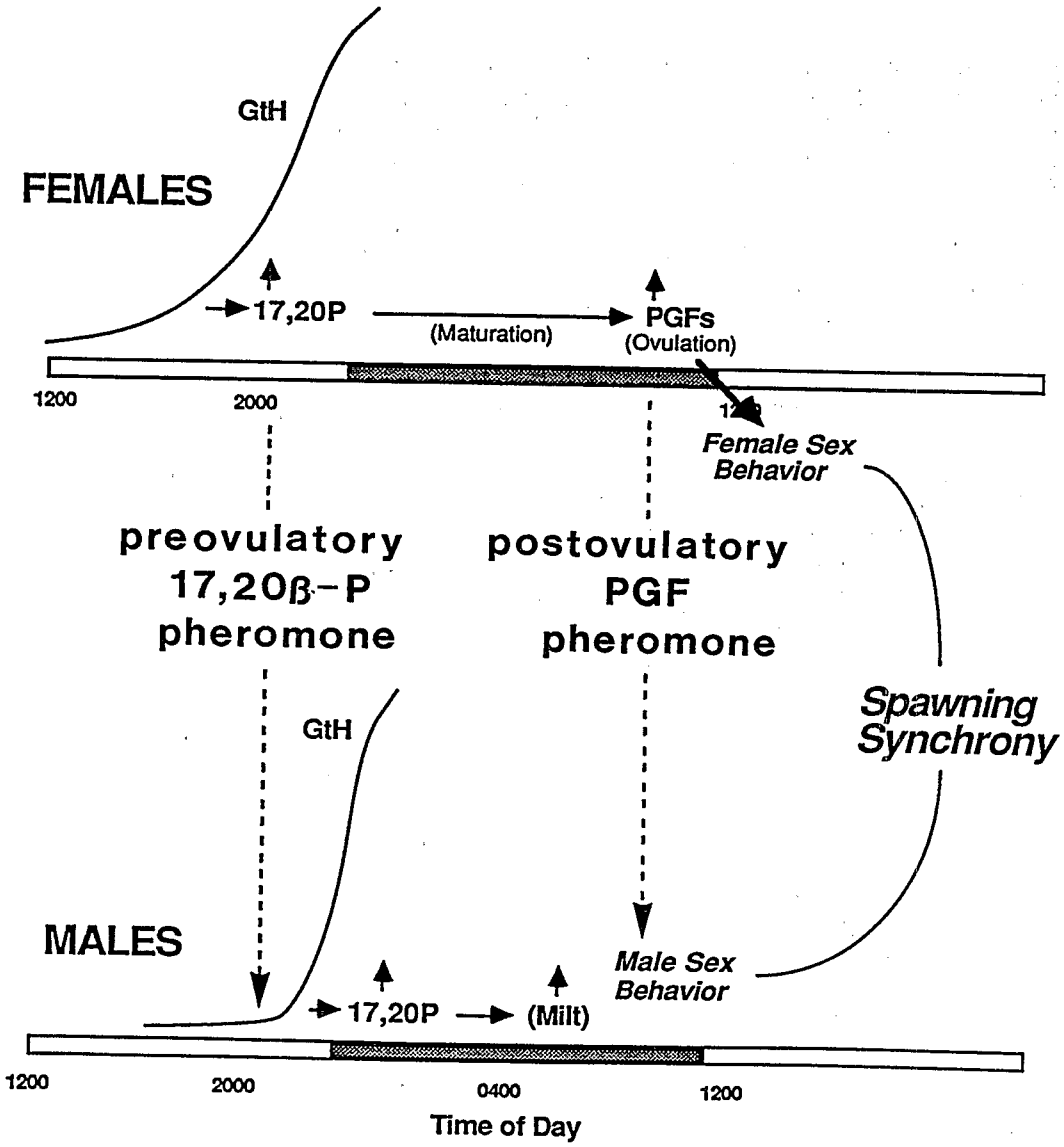

Figure 1. Schematic model depicting actions of two hormonal sex pheromones produced by periovulatory female goldfish. Preovulatory primer pheromone--a preovulatory surge in blood gonadotropin (GtH), triggered by environmental cues, stimulates ovarian synthesis of 17α20β-dihydroxy-4-pregnen-3-one (17,20β-P), which induces oocyte final maturation (resumption and completion of meiosis). 17,20β-P also is released to the water qhere it exerts a primer pheromone ennect on the male endocrine system, a rapid increase of blood GtH that stimulates testicular 17,20β-P synthesis, leading to increased releasable sperm within 2 to 4 hours. Postovulatory releaser pheromone--mature follicles rupture (ovulate) 10 to 12 hours after the GtH surge has begun. Ovulated oocytes in the reproductive tract then initiate and maintain the synthesis of F prostaglandins (PGFs), which enter the circulation and act within the brain to trigger female spawning behavior. PGFs and PGF metabolites also are released to the water where they function as a pheromone-triggering male sexual arousal.

(Stacey et al. in press), release of the steroid must be very rapid, an important feature if 17,20β-P concentrations in the water are to serve as an accurate indicator of impending ovulation. Nothing is yet known regarding the mechanism(s) of 17,20β-P release, although studies of other teleost species (Colombo et al. 1982) suggest that release in urine is likely.

Extracellular electrical recording from the goldfish olfactory epithelium (electro-olfactogram, EOG) has demonstrated that water-borne 17,20β-P is an extremely potent olfactory stimulant (1 pM threshold, Sorensen et al. 1987 and unpublished). EOG experiments testing more than 30 steroids have identified three types of steroid that are goldfish olfactory stimulants (Sorensen et al. 1987 and unpublished)--bile salts such as taurocholic acid; androgens such as androstenedione and testosterone; and "progestogens" related to 17,20β-P. Cross-adaptation experiments, in which the olfactory epithelium is first adapted to one odorant and then stimulated with another, indicate that the olfactory receptors responding to 17,20β-P are different from those responding to bile salts and androgens. Both the bile salts and androgens are considerably less potent than 17,20β-P, and the magnitude of the response induced by androgens is relatively small.

Of the progestogens tested, the three most effective are 17,20β-P, 17α,20β,21-trihydroxy-4-pregnen-3-one (17,20β,21-P), and 17α-hydroxyprogesterone (17-P), in decreasing order of potency. Because any other change to the 17,20β-P structure, or to its pregnane (A-ring-reduced) derivative, almost completely destroys activity (Sorensen, unpublished results), it seems likely that ovarian 17,20β-P does not require further metabolism in order to function as a pheromone. Because ovulatory goldfish release equivalent preovulatory surges of free 17,20β-P and conjugated 17,20β-P (likely 17,20β-P glucuronide), however, the possibility remains that conjugated 17,20β-P also may have pheromonal activity, (Stacey et al. in press). Unfortunately, 17,20β-P glucuronide is not available for testing. The goldfish olfactory system does not appear to detect glucuronides of estradiol, testosterone, or etiocholanolone (a 5β-reduced androgen) (Sorensen et al. 1987), despite the finding of Colombo et al. (1982) that the latter steroid attracts male goldfish.

When 17,20β-P is added to aquarium water to create supra-threshold concentrations, the blood GtH levels of males increase rapidly (within 15 min) and milt (sperm and seminal fluid) volumes increase within 2 to 4 hours (Dulka et al. 1987 and unpublished). Blood 17,20β-P levels of pheromone-exposed males also increase over this time period (Dulka et al. 1987), supporting the proposal that testicular 17,20β-P mediates GtH-stimulated sperm production (Ueda et al. 1985). Of a variety of steroids tested by addition to aquarium water, 17,20β-P is the most potent in inducing the GtH and milt increases, although related progestogens also are effective at higher concentrations. Androgens are completely ineffective in stimulating either GtH or milt volume increase, indicating their olfactory activity (as shown by EOG) is related to a different function (Stacey and Sorensen 1986 and unpublished). Both the GtH and milt responses to water-borne 17,20β-P are abolished either by complete section of the paired olfactory tracts or by selective section of the medial tracts (Stacey and Sorensen 1986, Dulka unpublished). These findings are consistent with a number of studies showing that olfaction is the dominant sensory mode for pheromone detection in fish and that pheromonal information

is conducted in the medial olfactory system (Stacey et al. 1986, Kyle et al. 1987).

Although simple addition of 17,20β-P to aquarium water consistently evokes rapid and dramatic increases in GtH level and milt volume (Stacey and Sorensen 1986, Dulka et al. 1987), it is likely that this "artificial" stimulation is not strictly comparable to that produced by natural interaction with a periovulatory female. For example, the GtH increase induced by chronic 17,20β-P exposure in aquaria reaches peak levels within 30 minutes and does not change for at least several hours (Dulka et al. 1987), whereas GtH levels of males exposed to the odor of females throughout the periovulatory period continue to increase until ovulation (Stacey et al. in press). Although there are many possible explanations for this difference between the effects of artificial and natural 17,20β-P exposure, we suspect that during artificial (chronic) exposure, adaptation of olfactory receptors makes the pheromone ineffective after a short period of time, whereas during natural exposure, the male is repeatedly stimulated by transient wisps of pheromone, each of which could independently lead to GtH increase. Indeed, we have calculated (Sorensen and Stacey in press) that the amount of 17,20β-P released by a female prior to ovulation is sufficient to produce only a small and very short-lived detectable odor plume, in which case males would not be chronically exposed to stimulatory 17,20β-P concentrations.

Also of concern in comparing the effects of artificial and natural 17,20β-P exposure is whether behavioral interaction with the female, or exposure to steroids other than 17,20β-P, normally contribute to the male's endocrine response. It seems unlikely that behavioral interaction with the preovulatory female is an important factor, because direct contact with periovulatory females induces no greater increase in GtH levels and milt volumes than does exposure to female odor (Stacey et al. in press). The potential involvement of steroids other than 17,20β-P is problematical, however, because periovulatory females also are known to release large quantities of 17-P (Van Der Kraak et al. 1989), which, although less potent than 17,20β-P, is capable of stimulating both GtH and milt increase (Stacey and Sorensen 1986 and unpublished).

Recently we have found that the male's response to water-borne 17,20β-P is affected by simultaneous exposure to water-borne androgens (unpublished results). The milt increase induced by 17,20β-P exposure is inhibited in a dose-dependent manner by water-borne testosterone and androstenedione. Androgens probably exert this effect by inhibiting GtH increase, but this has not yet been examined. The biological function for a potential inhibitory androgen pheromone is not clear because we do not understand the patterns of androgens released by male and female goldfish. At present, the simplest explanation would be that water-borne androgens inhibit inappropriate responses to the minor amounts of 17,20β-P that likely are released by males and nonovulatory females. Thus, only the preovulatory females release sufficient 17,20β-P to overcome the inhibitory effect of released androgens.

POSTOVULATORY RELEASER PHEROMONE

As shown in females of many teleost species (Liley 1982, Liley and Stacey 1983, Stacey et al. 1986), ovulated female goldfish release a pheromone that rapidly stimulates male courtship behaviors (Partridge et al. 1976, Sorensen et al. 1986). Our recent work (Sorensen, et al. 1988) indicates that this postovulatory releaser pheromone is comprised of $F_{2\alpha}$-series prostaglandins (PGFs).

A variety of experiments (see Liley and Stacey 1983, Stacey 1987, Stacey and Goetz 1982) indicate that prostaglandin $F_{2\alpha}$ first functions as a hormone that synchronizes spawning behavior with ovulation and, then, is released to the water where it acts as a pheromone. The presence of ovulated eggs in the reproductive tract stimulates synthesis of $PGF_{2\alpha}$, which then enters the bloodstream and is carried to the brain to trigger female sexual behavior. When oviposition is completed, $PGF_{2\alpha}$ synthesis decreases, $PGF_{2\alpha}$ is quickly removed from the circulation, and female sexual behavior ceases.

Sorensen et al. (1986) provided the first evidence that PGFs could function as female sex pheromones by showing that male goldfish exhibit similar behavioral responses to the odors of ovulated and $PGF_{2\alpha}$-injected females. However, because males exhibited no response when the $PGF_{2\alpha}$ was simply added to the water of the testing aquaria, they suggested that $PGF_{2\alpha}$ either was metabolized to an active form prior to release or stimulated production of an unrelated product with releaser pheromone effects. Further studies using EOG responses to evaluate the olfactory activity of PGs (Sorensen, et al. 1988) support the former possibility.

When a variety of prostaglandins (PGs) were tested as olfactory stimulants (Sorensen, et al. 1988), the most potent were found to be $PGF_{2\alpha}$ (100 pM threshold) and 15-keto-prostaglandin $F_{2\alpha}$ (15K-$PGF_{2\alpha}$; 1 pM threshold), a $PGF_{2\alpha}$ metabolite recently identified in goldfish (Goetz et al. 1987). Cross-adaptation studies indicate that these two PGFs stimulate separate classes of olfactory receptors, which are different from those that respond to 17,20β-P, bile salts, and amino acids (Sorensen, et al. 1988). PGs other than $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ also are effective in stimulating EOG responses, but only at much higher concentrations (1-20 μM).

Both ovulated and $PGF_{2\alpha}$-injected female goldfish release immunoreactive PGF to the water (Sorensen, et al. 1988). The rate of PGF release increases significantly at ovulation and continues to increase for at least 6 hours but rapidly declines to basal rates if ovulated eggs are removed by spawning or stripping. Because removal of eggs also abolishes female spawning behavior (Stacey 1987), it appears that a single mechanism, activated by the presence of ovulated eggs, produces the $PGF_{2\alpha}$, which first acts as a hormone to stimulate female sexual behavior and later acts as a pheromone to stimulate male sexual behavior.

Although there is as yet no direct evidence that ovulated female goldfish release 15K-$PGF_{2\alpha}$, there is strong indirect evidence that this is the case. For example, the potency of the odor of $PGF_{2\alpha}$-injected females (as measured by evoked EOG responses) is greater than would be expected if the $PGF_{2\alpha}$ had been

released without being metabolized, and similar to what would be predicted if the $PGF_{2\alpha}$ were converted to 15K-$PGF_{2\alpha}$ prior to release (Sorensen et al. 1988).

More recently, we (Sorensen et al. 1988, Sorensen et al. in press) have shown that both water-borne $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ stimulate the courtship behaviors exhibited by males exposed to the odors of ovulated or $PGF_{2\alpha}$-injected females (Sorensen et al. 1986). When PGFs were added to aquarium water, grouped males immediately decreased feeding activity and increased swimming activity and social contact with conspecifics. These studies also have confirmed and extended the findings of Kyle et al. (1985) that GtH levels of male goldfish increase during sexual interaction with $PGF_{2\alpha}$-injected females. In particular, experiments comparing the effects of PGF exposure on grouped and isolated males provide strong evidence that the pheromone does not increase GtH directly, but acts indirectly by stimulating sexual activity (Sorensen, et al. in press). Thus, the actions of water-borne PGFs are distinctly different from those of water-borne 17,20β-P, which induces equivalent reproductive responses in grouped and isolated males (Stacey and Sorensen 1986).

An unresolved and potentially important aspect of PGF pheromone function in goldfish is whether $PGF_{2\alpha}$ and 15K-$PGF_{2\alpha}$ perform the same or different functions. Although the presence of separate olfactory receptors for these PGFs suggests the potential for distinct effects, only qualitative differences in responses have so far been observed (Sorensen, et al. in press). We believe that by responding to two PGFs males are able to increase the distance over which they can locate ovulated females. For example, were males to employ only a single class of PGF olfactory receptor, saturation would occur as the male approached the female, with the result that no further increase in concentration would be detectable. By employing two classes of PGF receptors, with different sensitivities but similar central effects, males may extend the range of PGF concentrations over which a gradient can be detected.

FURTHER ASPECTS OF SEX PHEROMONE FUNCTION IN GOLDFISH

We are only beginning to understand the nature of sex pheromone function in goldfish; new pheromones, and new functions for identified pheromones, certainly await discovery. For example, the studies of Yamazaki and Watanabe (1979) suggest that sex-specific goldfish pheromones may be induced by estrogen and androgen treatment. Also, there are suggestions that the pheromonal function of 17,20β-P is more complex than originally expected. We recently have found that low concentrations of water borne 17,20β-P increase the occurrence of ovulation in goldfish (Sorensen and Stacey 1987 and unpublished), an effect consistent with the fact that 17,20β-P induces equivalent EOG responses in male and female goldfish (Sorensen et al. 1987). If this is a pheromonal effect, then the concept of 17,20β-P as a <u>female</u> sex pheromone will need to be revised. Indeed, because exposure to water-borne 17,20β-P increases 17,20β-P blood levels (and presumably 17,20β-P release) in male goldfish (Dulka et al. 1987), it is entirely possible that 17,20β-P is a bisexual pheromone, produced by, and acting on, both male and female. Such a pheromonal mechanism could synchronize the spawning of local populations.

HORMONAL PHEROMONES IN OTHER TELEOSTS

Our findings that goldfish sex pheromones are hormones provides strong empirical support for theoretical arguments (Doving 1976, Colombo et al. 1982) that fish commonly use released hormones for intraspecific reproductive synchrony. Although the generality of hormonal pheromones in fish cannot be assessed until more species are investigated, in all cases where the chemical identity of a fish sex pheromone has been proposed, the active substance is a sex hormone or metabolite (Lambert et al. 1986, Stacey et al. 1987, Sorensen and Stacey in press).

Although pheromonal effects of progestational steroids (i.e., 17,20β-P-like compounds) appear to have been studied only in the goldfish, Van Den Hurk et al. (1987) propose that glucuronated androgens released by male zebrafish (Brachydanio rerio) acts as a primer pheromone to trigger ovulation in the female. Another glucuronated androgen, etiocholanolone glucuronide, has been suggested to act as a releaser, attracting the ovulated female Gobius joso to the male's territory (Colombo et al. 1982). In fathead minnows, Pimephales promelas (Cole and Smith 1987), and milkfish, Chanos chanos (C.D. Kelley, C.S. Tamaru, and C.-S. Lee, Oceanic Institute, personal communication), male courtship responses are triggered by the odor of $PGF_{2\alpha}$-injected females and water-borne $PGF_{2\alpha}$, respectively. Together with the finding that PGFs induce EOG responses in arctic charr, Salvelinus alpinus (T. Sveinsson and T.J. Hara, Freshwater Institute, personal communication), these recent studies suggest that hormonal pheromones are widespread among teleosts.

PRACTICAL APPLICATIONS OF FISH SEX PHEROMONES

There are several reasons for considering the application of sex pheromones to control certain reproductive processes that presently are managed by hormone or drug injection. First, because pheromone treatment does not require the handling of fish, both labor costs and fish stress are reduced. Second, hormonal pheromone therapies will affect only fully mature individuals because they simply mimic natural triggers of endogenous reproductive events (ovulation, spermiation). It should be possible, therefore, to eliminate a difficulty often associated with injection therapies: determining whether individual fish are in a reproductive state appropriate for treatment. Third, present information suggests the level of technical expertise required to carry out pheromone treatment will be less than that required for treatment with exogenous hormones. Fourth, fish marketability is not likely to be affected by a treatment that applies a natural product exogenously, at concentrations lower than those present in the fish.

We believe that, in each teleost species, reproductive strategy and reproductive endocrinology work in concert to determine what sex pheromone functions will evolve (intersexual primer, intersexual releaser, territorial advertisement, etc.), and what hormones will be selected to play these roles (Sorensen and Stacey in press). Because the reproductive biology of many cultured cyprinids is broadly similar to that of the goldfish, we also believe that all these species use similar sex pheromone systems, and that the pheromonal effects we have demonstrated in goldfish, therefore, can be readily

applied to the reproductive management of cultured cyprinids. There seems no reason why 17,20β-P could not be used to stimulate spermiation of cyprinid broodstock. On the other hand, because cyprinid ovulation often is synchronized with daily photoperiod, further work on the ovulation inducing effect of water-borne 17,20β-P is required to establish the most effective time of exposure, and minimum duration of exposure required.

It also might be possible to advance gonadal development by elevating GtH levels with longterm progestogen treatment (e.g., daily pheromone pulses). Prostaglandin pheromones also may be useful in carp culture, one obvious application being the use of PGF-treated females as pheromone sources to segregate sexually mature males from mixed-sex schools or to attract and collect wild males for broodstock. Whether the inhibitory androgens have any practical application is not yet clear, although they may be useful agents for delaying spermiation and ovulation to extend the period of egg production.

The reproductive effects of hormonal sex pheromones in goldfish are dramatic and can be reliably induced with minimal expense and experience. If the same effects can be demonstrated in farmed cyprinids, a novel approach to the problem of controlled finfish reproduction may be at hand.

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EFFECT OF FEMALE-SPECIFIC PROTEIN COMPOUND TREATMENT ON HATCHING RATE OF FISH LARVAL AND FISH GONAD MATURATION (PROTEINS ENGINEER I)

by

Wang Hao and Liu Rongzhen

The effects of partially purified fish gonad protein on increasing oocyte maturation and hatching rate of fish larval were studied in adult freshwater and seawater fishes (*Hypophthalmichthys molitrix*, *Cyprinus carpios* L, *Ctenopharyngodon idellus*, *Mugil soiuy*, *Aristichthys nobilis*, *Parabramis perkinensis*, *Carassius auratus*, *Monopterus albus*).

In our work, female-specific protein compounds from fish gonads were isolated by using biochemical methods. All experimental fishes received intramuscular or intraperitoneal female-specific protein injections of 2 to 4 ml extractions for each fish in our experiment. After injection, at 3 to 7 days the hatching rate of fish larval were 89% and 90% in female *Aristichthys nobilis*, and the hatching rate of *Aristichthys nobilis* larval of control were 65% and 66%, respectively. The results of observation of all experimental female fish have increased the hatching rate by about 20%. Gonad weights of experimental fishes were higher than those of controls. Controls received injections of saline solution only. Our extracted female-specific protein from fish gonad was very effective. When it was used, it induced oocyte maturation and increased hatching rate of fish larval, as well as related phospholipids.

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TABLE 1. INJECTING (INTRAPERITONEAL) FEMALE-SPECIFIC PROTEIN SOLUTIONS AND THE EFFECTS ON OOCYTE GROWTH IN FEMALE *Carassius auratus*

No. of fish	Body wt, g	Gonad wt, g	Mean oocyte diameter, μm
2	167	15.4	750
3	172	20	780
15	170	19	775
16	190	15.8	790
17	148	14	780
18	141	9	805
19	198	16.5	821.4
20	200	16.5	821.4
21	209	17.5	857.7
Control			
6	124	13	400
4	129	4.7	450
7	147	9	560
9	196	9.5	688.6
10	189	6.60	610.5

TABLE 2. INJECTED FEMALE-SPECIFIC PROTEIN TREATMENT ON FEMALE *Mugil soiuy* AND THE EFFECTS ON HATCHING RATE OF FISH LARVAE

Experiment	Dose, ml	Fertilization rate, %	Hatching rate of fish larval, %
A	2	62.9	24
B	2	72.2	23.4
C	2	71.6	x
D	2	76	22.5
Control			
1	0	70.6	7.4
2	0	58.5	6.07
3	0	40	x

ANTIFREEZE PROTEIN GENES: PHYSIOLOGICAL REGULATION AND POTENTIAL VALUE TO THE GENETIC ENGINEERING OF FREEZE RESISTANT FISH

by
Garth L. Fletcher[1], Margaret A. Shears[1], Madonna J. King[1], Ming H. Kao[1], Peter L. Davies[2] and Choy L. Hew[3]

INTRODUCTION

The freezing point of most aqueous solutions depends upon the concentration of dissolved solute particles (colligative properties). The higher the concentration of solute particles the lower the freezing point. Thus distilled water freezes at 0°C, whereas the body fluids of most fishes freeze at -0.5 to -0.8°C depending on their salt content. This means that fishes inhabiting fresh water are in no danger of freezing, unless, of course the water turns solid. Seawater has a salt concentration that is approximately three times that of fish body fluids. Therefore, its freezing temperature ranges from -1.7 to -2°C, depending on salinity.

Since the seawater temperatures in the polar oceans and along the Northeast Atlantic coast of Canada approach freezing for at least part of the year, why do not fishes inhabiting these waters freeze and die? Scholander and his colleagues first attempted to answer the question by studying a number of marine fish inhabiting the coast of Northern Labrador (Scholander et al. 1957, Gordon et al. 1962). They made two important observations. Some fish do survive supercooled; that is, at temperatures below their freezing point. If they come into contact with ice, however, they immediately freeze and die. Therefore, it is the combination of supercooling and ice contact that is lethal.

The second observation made by Scholander's group was that the body fluids of some fish had the same freezing point as seawater. They termed the responsible solute "antifreeze." The early work of DeVries and Feeney (DeVries et al. 1970) established that the antifreezes were polypeptides. Since that time, four distinct classes of antifreeze polypeptides differing in

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carbohydrate content, amino acid composition, protein sequence, and secondary structure have been isolated from the blood sera of a diverse group of marine teleosts. These proteins are synthesized in the liver for export to the blood, which distributes them throughout most of the extracellular space where they effectively protect the fish from freezing at temperatures as low as the freezing temperature of seawater (-1.8°C) (Hew and Fletcher 1985, Scott et al. 1986, Davies et al. 1988).

WINTER FLOUNDER ANTIFREEZE PEPTIDES

We have been studying the antifreeze polypeptides (AFP) of the winter flounder (Pseudepleuronectes americanus) (Figure 1) for the past 15 years (Hew and Fletcher 1985, Davies et al. 1988). This marine flatfish inhabits shallow inshore coastal waters of much of the Northeast Atlantic coast of North America and many populations are exposed to freezing temperatures (<-0.7°C) and ice every winter (Fletcher et al. 1985).

The AFP in winter flounder consist of a family of at least seven independently acting polypeptides ranging in size from 3300 to 4500 daltons. The two major AFP (3300 daltons) found in the blood plasma have been sequenced completely and one of them has been crystallized to reveal that they are amphiphilic α helices. The majority of the hydrophilic amino acid side chains project along the length of one side of the helix and the opposite side is predominantly hydrophobic (Figure 2) (Davies et al. 1982; Pickett et al. 1984; Fourney et al. 1984a; Hew et al. 1986 a,b; Yang et al. 1988). It is believed that the hydrophilic residues bind (hydrogen bond) to embryonic ice crystals, thus preventing their growth by blocking the further addition of water molecules (DeVries and Lin 1977). Yang et al. (1988) recently refined this model and elaborated on the mechanism by which the antifreeze peptides complex with ice.

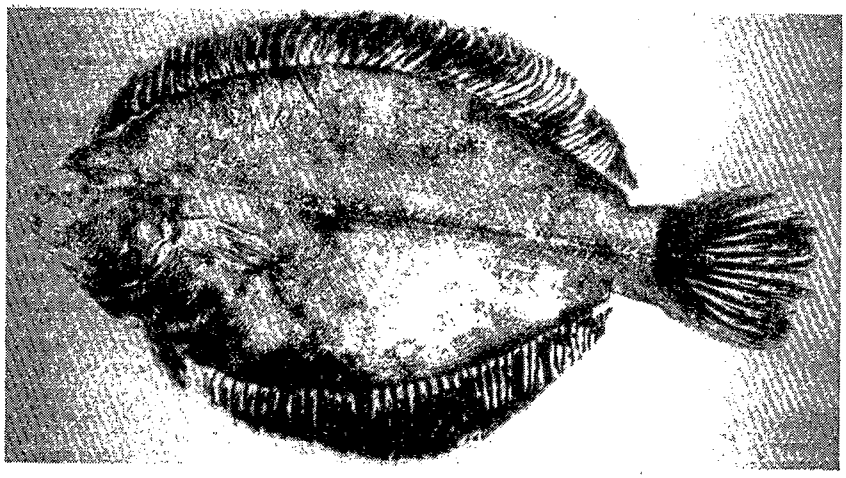

Figure 1. Gyotaku print of a 35-cm winter flounder (Pseudopleuronectes americanus)(artist Ron Fourney).

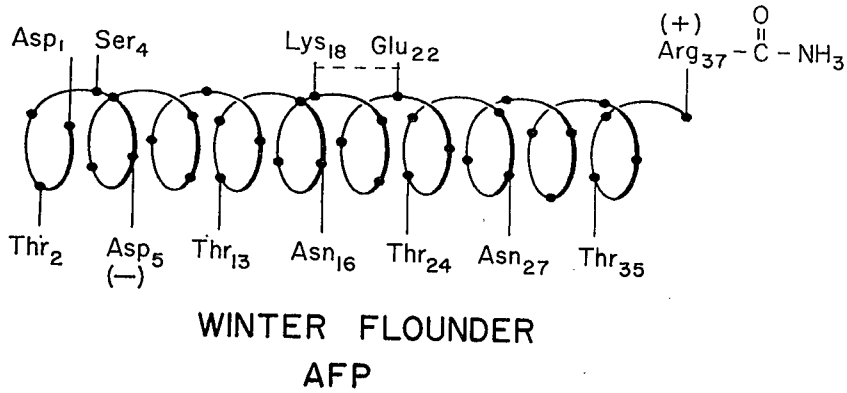

Figure 2. Structure of winter flounder AFP. [The alpha-helical secondary structure of the AFP is indicated by the coil. Residues (•) with hydrophilic side chains are indicated and those that are postulated to interact with the ice lattice project downwards. The broken line between lysine and glutamate indicates an intramolecular salt bridge. The molecule is about 50 nm long.]

In Newfoundland populations of winter flounder, mature AFP appear in the blood plasma in November when the water temperature approximates 4 to 6°C, reach peak values by January, and disappear during May when the temperature begins to rise above 0°C (Figure 3) (Fletcher 1977). These seasonal changes in plasma AFP levels are accompanied by concomitant changes in the winter flounder's resistance to freezing. Moreover, the winter increase in freezing resistance over that observed during the summer is identical to the winter increase in plasma antifreeze activity (Figure 4) (Fletcher et al. 1986).

The environmental and physiological factors regulating the annual AFP cycle in winter flounder have been reviewed in detail by Davies et al. (1988) and Fletcher et al. (1988a). All of the available data suggest that the annual cycle is endogenous, with photoperiod acting via the pituitary gland to regulate the precise time of onset of AFP production in the Fall (Fletcher 1977; Fourney et al. 1984 b,c). Water temperature does not appear to play a major role in controlling the time of AFP production, but it does affect the rate of AFP disappearance from the blood. At the water temperatures that prevail in Newfoundland in the Spring (March and April) (0 to -1°C) plasma AFP levels remain at winter values (February) despite the fact that AFP synthesis has stopped. Only when the water temperatures increase above 0°C will AFP levels decline (Fletcher 1977, Hew et al. 1986b). This means that the fish are protected from freezing even when there is an unusually prolonged winter.

ANTIFREEZE PEPTIDES AND ATLANTIC SALMON CULTURE

Approximately 7 years ago, Dr. Arnie Sutterlin brought to our attention the economic desirability of culturing Atlantic salmon in sea cages along the Atlantic coastline of Canada. No evidence for the presence of AFP has been found in any salmonid; therefore, the problems associated with this endeavour were obvious to all of us: the icy marine environment was lethal to salmon (Fletcher et al. 1988b). Because the only fish capable of surviving in such an environment were those possessing antifreeze polypeptides, we decided to

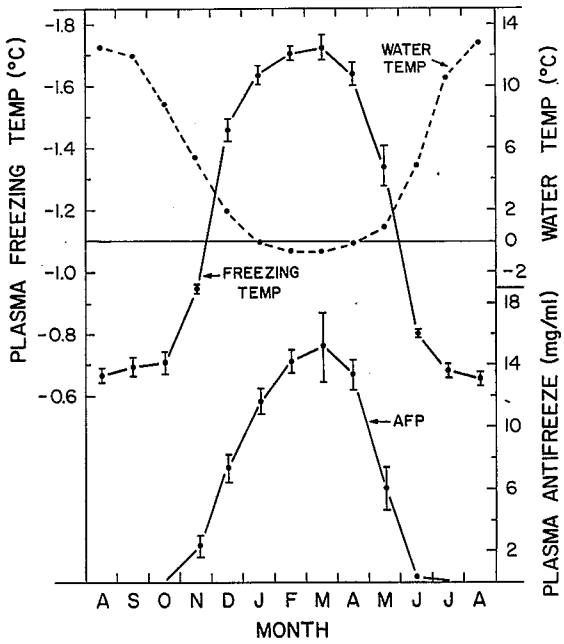

Figure 3. Annual cycles of plasma freezing temperatures and plasma antifreeze peptide (AFP) levels in Newfoundland winter flounder. [All values plotted as means ± one standard error (N=5 to 10).]

attempt to make Atlantic salmon more freeze resistant by giving them a set of antifreeze genes.

DO ANTIFREEZE PEPTIDES CONFER FREEZING RESISTANCE TO FISH?

Because we were embarking on a project to improve the freezing resistance of Atlantic salmon by winter flounder gene transfer, we needed direct evidence that the AFP was not species specific and that it would increase the freezing resistance of salmon.

The lethal freezing temperature of both seawater-acclimated Atlantic salmon and rainbow trout was approximately -0.75°C (Figure 4) (Fletcher et al. 1988b). When AFP purified from the blood plasma of winter flounder were injected into the rainbow trout, their freezing resistance increased in direct proportion to blood AFP levels (Figure 4) (Fletcher et al. 1986). These results indicate that Atlantic Salmon can be engineered to tolerate subzero water temperatures and ice if winter flounder AFP genes can be inserted into the salmon genome and expressed in physiologically significant quantities.

WINTER FLOUNDER ANTIFREEZE GENES

During the course of our studies on the antifreeze peptides and their regulation in winter flounder, the genes coding for these peptides were

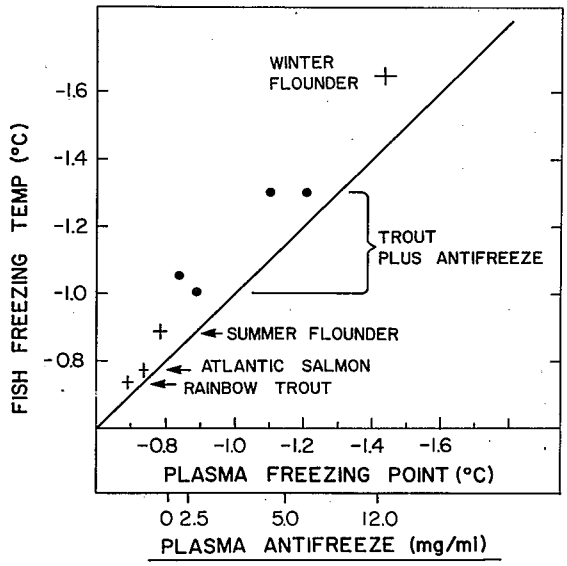

Figure 4. Relationship between lethal freezing temperatures and plasma freezing temperatures. [Lethal freezing temperatures were determined on seawater-adapted fish by gradually lowering the temperature of the aquaria with the use of crushed seawater ice. "Winter" flounder are winter flounder acclimatized to winter conditions. "Summer" flounder are winter flounder acclimated to summer conditions. Experiments on salmon and rainbow trout were carried out during late winter. Crosses represent means ± one standard error. Solid dots represent individual samples from rainbow trout injected intraperitoneally with winter flounder AFP. The diagonal line indicates the water temperature at which the fish should die as predicted from the plasma freezing points. Data summerized from Fletcher et al. 1986, 1988.]

isolated (Davies et al. 1984). The functional AFP gene is 1 kilobase (kb) long and contains a 600 base pair (bp) intron between two exons. Genomic Southern blots show that there are approximately 30 to 40 genes per haploid genome. The majority of these AFP genes (>20) are present in 7 to 8 kb elements of DNA, which codes for the major AFP found in flounder blood plasma (Scott et al. 1985).

In preparation for our gene transfer experiments, one of the genomic tandem (Bam H1) repeats (7.8kb) (subclone 2A-7) was cloned into the Bam H1 site of plasmid pUC-9 using E. coli as the host to produce large quantities of the gene (Figure 5). The plasmid containing the antifreeze protein gene was harvested from the E. coli, purified, and linearized for injection by digestion with Eco R1 (Fletcher et al. 1988c). Because the gene used for injection was an entire tandem repeat of the winter flounder antifreeze gene, it should contain all of the DNA sequences responsible for controlling its expression (Scott et al. 1985).

TECHNIQUES FOR GENE TRANSFER

From the outset of our studies, we have concentrated on microinjection techniques to transfer the antifreeze genes to salmon eggs. The most

effective place to inject any foreign gene is directly into the nucleus. For a number of reasons, however, this is not easy to do with salmonid eggs. Although salmon eggs are very large (5mm diameter) they are also opaque, making it impossible to see the nuclear area. In addition to this, once the eggs are fertilized in water, the chorion separates from the perivitelline membrane and hardens, making it very difficult to penetrate with fine glass needles. This is unfortunate, for once the perivitelline space has formed, the germinal disc is always on top of the yolky egg where it can be readily located for microinjection.

Salmon eggs are fertilized by the entry of a sperm through the micropyle, which is located close to the dividing egg nucleus (Figure 6) (Ginsburg 1968, Riehl 1980). Thus it was evident that the best means of locating the female nucleus was to find the micropyle. Although it took us a number of spawning seasons to perfect this technique, we can now do it with relative ease under a dissecting microscope using reflected light. Once the micropyle is located, a 3 to 5 μm (outside diameter) glass needle can slide without resistance into the germinal vesicle area of the egg.

One potential problem with using the micropyle as an injection route is that it may become altered in such a manner as to interfere with the normal fertilization process. Thus we decided to carry out the microinjections after fertilization.

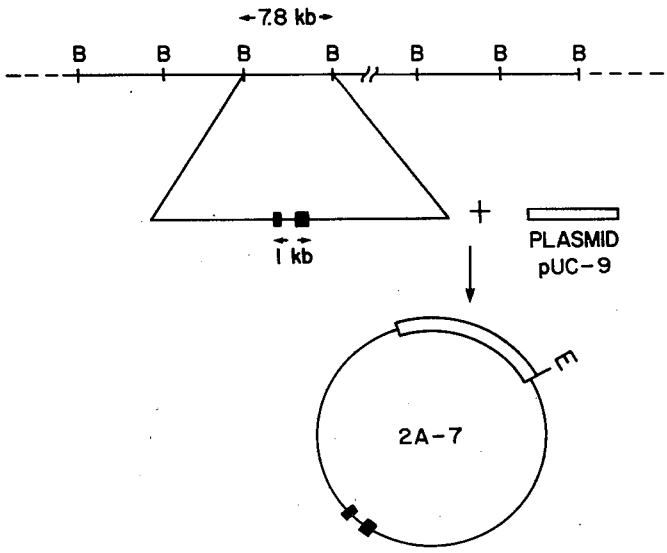

Figure 5. Schematic diagram of tandemly repeated antifreeze gene locus in winter flounder. [B=Bam HI site, E=EcoR1. The discontinuity indicates the varied number of repeats in a gene cluster. The AFP gene (1 kb long) is indicated in the enlargement showing its single intron and two exons. One of the tandem repeats was cloned into the Bam HI site of the plasmid Puc 9. The plasmid containing the AFP gene (clone 2A-7) was harvested from E. coli and linearized for injection using restriction endonuclease EcoR1.]

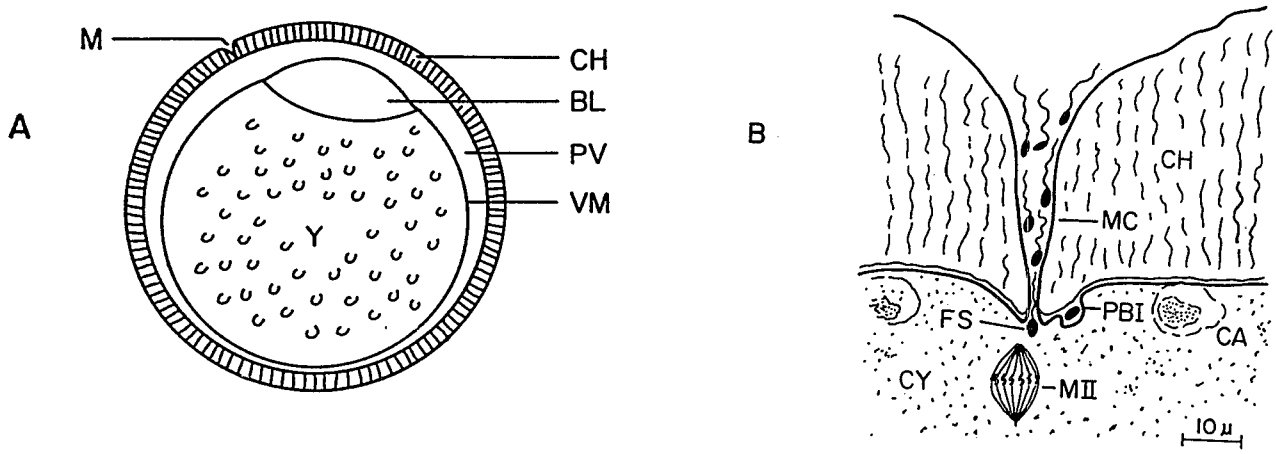

Figure 6. Schematic of fertilized salmon egg (A) and micropyle (B). [A--BL=blastodisc, CH=chorion, M=micropyle, PV=perivitelline space, VM=vitelline membrane, and Y=yolk. B--CA=cortical alveoli, CH=chorion, CY=cytoplasm, FS=fertilizing spermatozoa, MII=metaphase of second maturation division, MC=micropilar canal, and PBI=first polar body. Some of the ideas for these drawings are from Ginsburg (1963, 1968).]

Once fertilized, the chorion immediately begins the water hardening process, thus the time available to use the micropyle for injection is relatively short. To extend this time, we followed up on Ginsburg's (1963) technique of fertilizing the eggs in a salmon ringer solution. When this procedure is used, the eggs are fertilized but not activated and the water hardening process is delayed until the eggs are placed in fresh water.

The injection apparatus consists of a 3 to 5 μm (O.D) glass needle driven by a micromanipulator. The volume of DNA solution injected (2 to 3 nl) was regulated using short bursts (100 to 1000 ms) of N_2 at 200 kPa. The timing of the bursts was controlled using a Grass Stimulator. The eggs were injected with approximately 1 X 10^6 copies of the linearized antifreeze gene plus plasmid (pUC-9) through the micropyle within 2 hours of fertilization, and incubated in freshwater at 8°C.

ANTIFREEZE GENE TRANSFER

Details of the antifreeze gene transfer experiments have been published (Fletcher et al. 1988c). Of the 1800 Atlantic salmon eggs injected with the antifreeze genes, approximately 80% survived to hatching. This survival rate was the same as that of uninjected eggs.

Eight months after injection 30 fingerlings (1 to 2 g each) were collected for DNA analysis. Genomic DNA was isolated from individual fingerlings as described by Scott et al. (1985). These DNAs were screened for the presence of the winter flounder antifreeze gene using genomic Southern blotting procedures (Southern 1975). In this technique, the high molecular weight DNA was cut into smaller fragments using restriction enzymes and the resulting solution was electrophoresed in 0.7% agarose gels. The DNA fragments, now separated according to size, then were transferred to

nitrocellulose and probed for the presence of antifreeze genes using a short ^{32}P labelled segment (2.7 kb) of DNA containing the complete antifreeze gene (Figure 7). If winter flounder antifreeze gene sequences are present in the salmon DNA, the ^{32}P labelled probe will bind to them and they can be localized using autoradiography.

EVIDENCE FOR ANTIFREEZE GENE TRANSFER TO ATLANTIC SALMON

A preliminary screening demonstrated that the DNA from 2 of the 30 salmon fingerlings tested contained the antifreeze genes. A more detailed analysis of these two positive DNAs (No. 26 and No. 36) along with the DNA of one negative control (No. 45) was carried out in order to be certain that the complete antifreeze gene was integrated into the salmon genome (Fletcher et al. 1988c).

When the salmon DNA was not cut with restriction enzymes, the ^{32}P-labelled probe only hybridized to large molecular weight DNA, indicating that the injected genes were present within the salmon genome rather than free within the cell. When the DNA was cut with restriction endonucleases Sst 1 and Bam HI, the probe hybridized to 2.7 and 7.8 kb DNA segments, respectively. Segments of this size are what would be predicted if the injected DNA gene sequence was cut with the same enzymes. When the salmon DNA was cut with restriction enzyme Hind III, the probe hybridized to DNA fragments of 9.4 kb and longer in one of the two positive salmon and 13.5 kb and longer in the other. Because the gene used for injection had only one site at which this enzyme could cut, the other cut had to be within the salmon DNA itself. Because we cannot predict where this cut would be, the size of the fragment would be at least 7.8 kb (the length of the injected winter flounder gene) and have a variable upper length (Figure 7) (Fletcher et al. 1988c).

The minor bands in the Bam HI and Hind III lanes of 26 and 36 possibly represent cleavage products of 2A-7 that have been independently incorporated into the salmon genome. In this situation, one would expect to see fewer of these accessory bands in the Sst I lanes than in the other two digests, simply because there is less chance of the breaks in 2A-7 occurring within the central 2.7-kb Sst I fragment than within the 7.8-kb Bam HI fragment. When the overall hybridization signal derived from the 30 to 40 genes in the Sst I digested winter flounder standard (Figure 7) is considered, it is clear that more than one copy of the AFP gene had been incorporated into the transgenic salmon. The exact number of copies per cell must await further research.

The integration frequency of the injected genes in the present study (2 out of 30; 6%) appears to be similar to that observed by other investigators using microinjection procedures. Hammer et al. (1985) found an integration frequency of 1 to 13% for several mammals. Maclean et al. (1987) reported a 5% integration frequency of rat growth hormone-mouse metallothionein fusion gene in the genome of rainbow trout. Similarly, Dunham et al. (1987) demonstrated, using restriction analysis, the successful integration of a mouse metallothionein-human growth hormone fusion gene in 2 out of 10 (20%) channel catfish. Ozato et al. (1986) found evidence to suggest a 30% (10 of 30) integration frequency of a chick δ-crystalline gene construct. McEvoy et al. (1988) using dot blot hybridization procedures suggest that 2 out of 15

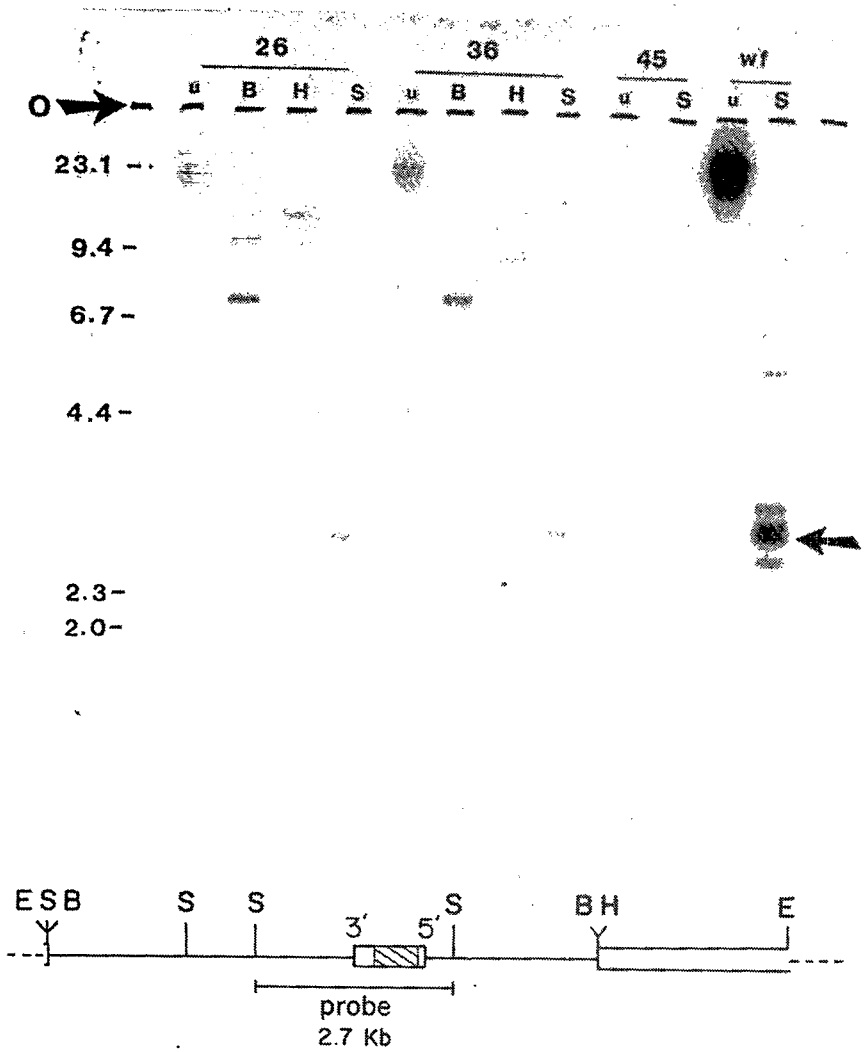

Figure 7. Genomic Southern blots of salmon DNAs. [26, 36, and 45 represent individual salmon. WF=DNA from winter flounder. U=undigested DNA. B,H, and S represent DNAs digested with restriction enzymes Bam HI, Hind III, and Sst I, respectively. The origin of the gel (O) is indicated by the arrow, and the length (kb) of DNA standards are indicated below it. On the right-hand side, the arrow indicates the point of migration of the AFP gene containing Sst I fragment of flounder DNA and Sst I fragment of 2A-7. The schematic diagram represents a restriction map of the integrated, linearized plasma 2A-7. Salmon genomic DNA is indicated by the broken line. The pUC-9 section of 2A-7 is indicated by the double lines. The 1-kb gene is represented by the small rectangular box. The open areas of the box are the exons (AFP coding sequences) and the hatched area is the intervening sequence (intron). The cleavage sites of restriction enzymes Bam HI (B), Sst I (S), and Hind III (H) are marked. The 2.7-kb Sst I fragment used as a radioactive probe is underlined. This figure is produced from a combination of Figure 1A and 2 of Fletcher et al. (1988c).]

(13%) Atlantic salmon embryos (14 weeks old) contained detectable levels of the E. coli β galactosidase-mouse metallothionein promoter fusion gene. Zhu et al. (1985) reported that 50% (3 out of 6) of their goldfish injected with a human growth hormone gene construct were transgenic; Chourrout et al. (1986) claimed an integration frequency of 75% by injecting a human growth hormone cDNA sequence into rainbow trout eggs.

An excellent study carried out by Stuart et al. (1984) examined 547 adult zebrafish that had been injected as embryos with a linearized bacterial plasmid (pSV-hygro) and found that only 5% of them retained the foreign DNA. The amount of foreign DNA present averaged less than one copy per cell. When 20 of their positive (transgenic) fish were crossed to uninjected control fish, only one consistently transmitted the foreign sequences to its offspring. One of their F_1 progeny was crossed with a control fish to yield an F_2 generation of which 50% contained the foreign DNA sequence. Their experiments demonstrate unequivocally that injected DNA can be integrated into the fish genome to give rise to a stable breed of transgenic fish.

ANTIFREEZE GENE EXPRESSION

We are screening the blood of potentially transgenic salmon for the presence of antifreeze peptides using immunoblotting procedures (Burnette 1981). Samples of blood plasma, approximately 10 μl, are analyzed on a 15% acrylamide sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose paper. Immediately after the proteins are transferred, the nitrocellulose sheet is incubated first in a solution containing bovine serum albumin and then in a solution containing rabbit anti-flounder AFP antibodies. The antibody-AFP complex is located on the nitrocellulose sheets by hybridization with ^{125}I-protein A followed by autoradiography (Hew et al. 1986).

The results obtained from screening blood plasma collected from 46 salmon fingerlings during February 1986 indicated that three of them contained low but detectable levels of winter flounder AFP. The immunoblotting results from one of the three AFP-positive salmon are illustrated along with several AFP-negative fish in Figure 8.

The molecular mass of the presumptive AFP found in the salmon plasma was approximately 6 kd, twice that of mature AFP found in winter flounder (3.3 kd) (Figure 8). An explanation for this observation comes from our knowledge of AFP gene expression in winter flounder. The winter flounder AFP gene used in this study codes for an 82 amino acid pre-proAFP (Figure 9) (Pickett et al. 1984). The 23 amino acid pre-sequence is cleaved off intracellularly and the proAFP is secreted into the blood where the pro-sequence is removed within 24 hours to yield a 37 amino acid mature AFP. The molecular size of the AFP-immunoreactive protein found in salmon plasma is essentially the same as that of the pro-AFP (6kd) isolated from flounder. This suggests that, although the Atlantic salmon can properly express the inserted winter flounder gene and secrete the proAFP into the blood, it lacks the necessary (enzyme) systems to process the pro-AFP to mature AFP. A similar result has been obtained in transgenetic Drosophila melanogaster for which only proAFP was found in the hemolymph (Rancourt et al. 1987).

Studies by Hew et al. (1986b) have demonstrated that proAFP isolated from winter flounder liver possess significant antifreeze activity (Figure 10). Thus although the proAFP are less effective than mature AFP at depressing the freezing temperature, they nevertheless would be capable of conferring increased freezing resistance to salmon.

We currently are attempting to repeat our observations of AFP gene expression by screening all (>700) of our individually tagged, potentially transgenic salmon. In this set of observations, DNA will be extracted from the red blood cells of fish with detectable plasma levels of AFP and analyzed for AFP genes using genomic Southern blotting procedures. All salmon showing good evidence for the presence of AFP genes and their expression will be selected for breeding experiments to determine their inheritability.

HOW CAN AFP GENE EXPRESSION IN SALMON BE IMPROVED?

It is evident from our preliminary screening that transformed salmon express the winter flounder AFP genes and secrete the proprotein into the blood. If these results are substantiated by further analysis, it appears that the level of expression and secretion of the proAFP will be low and not sufficient to confer a significant increase in the salmon's ability to resist freezing. Because blood plasma AFP concentrations of 10 mg/ml will be required for winter survival of sea-cage-farmed salmon in most locations along

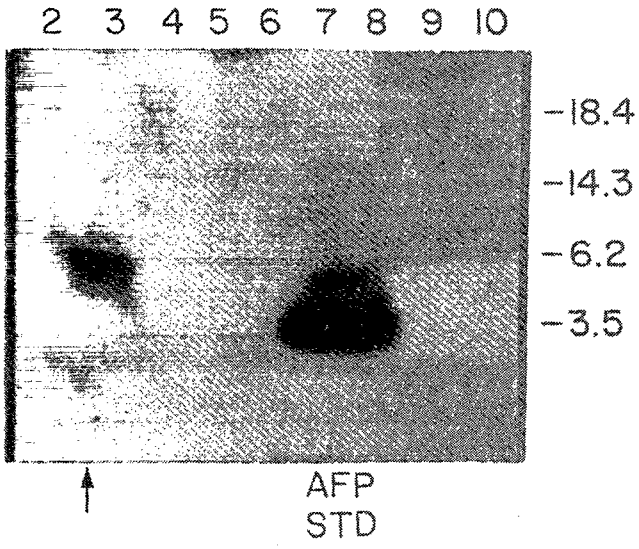

Figure 8. Immunoblots of Atlantic salmon plasma. [Blood plasma from Atlantic salmon that developed from fertilized eggs injected with winter flounder antifreeze genes (clone 2A-7) were analised on a 15% acrylamide SDS-PAGE and transferred to nitrocellulose paper. The paper was incubated with anti-AFP antibodies followed by ^{125}I lableled protein A. Lane 7 AFP standard. All other lanes contain salmon plasma. The arrow under lane 3 indicates an AFP-immunoreactive protein of approximately 6 kd. Numbers on the right side of the gel indicate the location of molecular weight markers.]

MET ALA LEU SER LEU PHE THR VAL GLY GLN LEU ILE PHE LEU
23
PHE TRP THR MET ARG ILE THR GLU ALA SER PRO ASP PRO ALA

ALA LYS ALA ALA PRO ALA ALA ALA ALA ALA PRO ALA ALA ALA
44
ALA PRO ASP THR ALA SER ASP ALA ALA ALA ALA ALA ALA LEU

THR ALA ALA ASN ALA ALA ALA ALA ALA LYS LEU THR ALA ASP
82
ASN ALA ALA ALA ALA ALA ALA ALA THR ALA ARG GLY

PRE PRO AFP = 82 AA

PRO AFP = 59 AA

MATURE AFP = 37 AA

Figure 9. Amino acid sequence of the antifreeze precursor from winter flounder (HPLC component 6). [The underlined region indicates the sequence of the mature protein (Hew et al. 1986).]

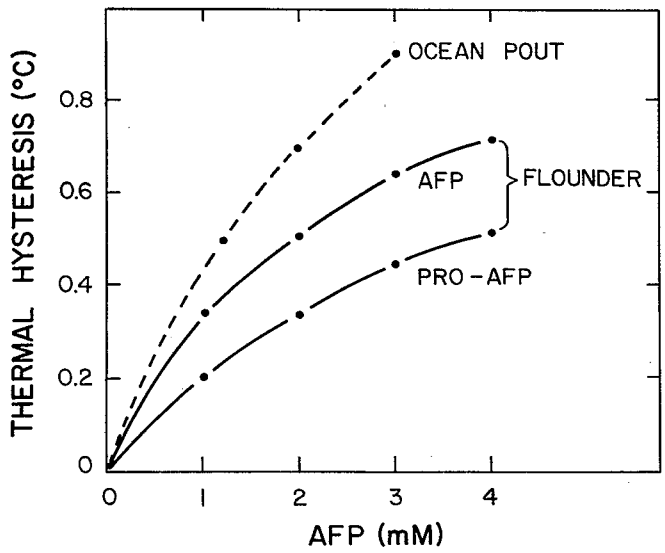

Figure 10. Antifreeze activity of winter flounder pro-AFP, winter flounder mature AFP, and ocean pout AFP. [Thermal hypteresis is a measure of antifreeze activity. Data summarized from Hew et al. (1986) and Kao et al. (1986).]

the East coast of Canada, the expression of these exogenous genes will have to be improved.

Studies of the antifreeze genes and their expression in natural populations provides us with a number of clues as to which way to proceed. Our research on the antifreeze peptides in a broad variety of taxanomic groups has led us to suggest that they evolved relatively recently in response to the appearance of seawater ice (Scott et al. 1986). In many species, the genes coding for these antifreezes are amplified, suggesting intense selective pressure to produce large amounts of protein. Recently we have found evidence that the degree of gene amplification (gene dosage) can be correlated directly with the level of AFP produced (Fletcher et al. 1985, Scott et al. 1988a, Hew et al. 1988).

Because gene amplification appears to be a common mechanism by which fish have increased their circulating AFP levels, it seems reasonable to look for ways to increase AFP gene copy number in salmon.

The winter flounder DNA sequence used in the present study represents one of the tandemly repeated genes; therefore, it may well contain elements responsible for gene amplification. If such sequences are present, they could lead to a similar expansion of AFP gene number in transformed salmon. Our breeding experiments with the transformed salmon should determine whether this will occur. These experiments, however, may take some time for Stuart et al. (1988) found, using zebrafish, that it took until the F_2 generation to produce a stable line of transgenic fish.

It does not seem wise to rely entirely on the winter flounder AFP genes to transfer freezing resistance to salmon. For one thing, the proAFP produced by the transformed salmon is a less active antifreeze than the mature protein. Furthermore, we recently have found that the winter flounder AFP genes are regulated by growth hormone (Vaisius et al. 1988). Growth hormone must be absent, or at low levels, in the plasma before AFP genes can be transcribed. If this regulatory system is passed on to the salmon, there may be conditions under which growth hormone levels may not be low enough during winter for transferred AFP genes to be transcribed.

Recently, we have been considering the potential value of wolffish and ocean pout AFP genes as candidates for gene transfer to salmon. Wolffish (Anarhichas lupus) and Ocean pout (Macrozoarces americanus) are members of different families from the suborder Zoarcoidea. Both species produce high concentrations (20 to 30 mg/ml) of AFP (6 kd), which differ markedly from that of the winter flounder in that they are not α helical and do not possess an abundance of alanine (Hew et al. 1984, 1988; Scott et al. 1988b).

There are four reasons why the AFP from wolffish and ocean pout may be useful for gene transfer. First, both species appear to be expressing their AFP genes constitutively for they maintain significant plasma concentrations all year round (Fletcher et al. 1985 and unpublished data). Furthermore, plasma AFP levels, at least for the ocean pout, are controlled to some degree by water temperature. This is not the case with winter flounder where AFP genes are controlled by the pituitary. Second, the fact that they are not

biased, in the way winter flounders are, to the use of alanine might make it easier for the salmon to synthesize them in large quantities. Third, neither the wolffish nor the ocean pout produce pro AFP. Therefore, no special mechanisms would be required by the salmon to produce fully efficient mature antifreeze. Fourth, on a molar basis, the ocean pout AFP, and most likely the wolffish AFP, are approximately 40% more active than the mature winter flounder AFP (Figure 10).

The AFP genes from the wolffish and the ocean pout are encoded in large multigene families of approximately 80 and 150 members, respectively (Hew et al. 1988, Scott et al. 1988b). Detailed studies of AFP gene organization in the wolffish indicate that two-thirds of the 80 to 85 genes are organized in 8 kb tandem repeats, each of which contains two genes in inverted orientation. These repeats are clustered in groups of at least six (Scott et al. 1988b). A restriction map of one genomic clone (Wr4A) containing two-and-one-half repeats (five AFP genes) is illustrated in Figure 11. We are now injecting this 16 kb stretch of wolffish DNA into the salmon eggs. By using this relatively long sequence, we are not only attempting to directly increase the AFP gene copy number incorporated into the salmon genome but are also increasing the potential for gene expansion or amplification by unequal crossing over during meiosis (Hew et al. 1986a).

In summing up, it is apparent that, as we learn more about the regulation of antifreeze genes and their expression in natural populations, we will devise more appropriate gene constructs for engineering freeze-resistant fish. Although we look forward to the ideal result of finding that all transgenic fish produce enough antifreeze protein to survive down to the freezing point of seawater, it is more realistic, however, to anticipate that there will be a more limited expression of the transferred genes and that the production of freeze resistant salmon will have to rely on appropriate selection and mating techniques.

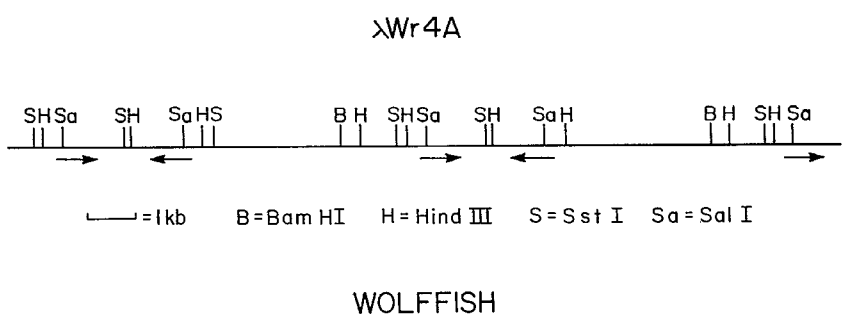

Figure 11. Restriction map of a wolffish genomic clone containing five antifreeze protein genes. [Arrows indicate positions of the genes and the directions of transcription. Redrawn from Scott et al. (1988).]

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BIOENERGETICS MODELING OF FISH GROWTH

by
Cui Yibo[1] and R. J. Wootton[2]

INTRODUCTION

Modeling the growth of fish is of great interest to fish biologists. Adequate growth models are needed for predicting the production of fish populations, which is important in the management of fisheries and for an understanding of the role of fish populations in aquatic ecosystems.

A popular approach to modeling fish growth is to regard the growth rate as a function of body size, such as the von Bertalanffy model. This approach does not explicitly consider the effects of causal factors. The model only reflects the growth of the fish under the average conditions of the water body concerned; it is descriptive rather than predictive.

Bioenergetics modeling has provided a promising alternative for predicting fish growth in varying environments. This approach considers the effects of causal factors--usually rate of food consumption, water temperature and body size--on each component of the energy budget of fish and integrates these effects to predict a growth rate. Bioenergetics models have been applied to a number of fish species (Kitchell et al. 1974, 1977; Kitchell and Breck 1980; Rice et al. 1983; Stewart et al. 1983; Diana 1983; Bevelhimer et al. 1985; Cuenco et al. 1985a,b,c; Stewart and Binkowski 1986) and have been applied to natural systems (Cochran and Rice 1982, Hurley 1986, Kitchell and Crowder 1987, Carline 1987). There has not been much improvement in the structure of the model, however, since it was first developed over a decade ago. Before we can be sure of extensive applications of this approach, the assumptions adopted in the model need to be justified, and the model has to be validated against data from independent, rigorous experiments.

The purpose of this study was to evaluate the use of bioenergetics models for predicting fish growth. A bioenergetics model was developed for the European minnow, Phoxinus phoxinus (L.). Using this model, the impacts of some assumptions commonly adopted in other bioenergetics models were assessed. Data from an independent experiment was used to validate the model.

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METHODS AND RESULTS

MODEL DEVELOPMENT

According to the energy budget of fish, growth can be expressed as:

$$G = C-F-U-SDA-Rs-Ra$$

where G is growth, C is food consumption, F is fecal production, U is excretion, SDA is specific dynamic action, Rs is standard metabolism, and Ra is activity metabolism (Stewart et al. 1983). In the model, each of the components on the right side of the equation is regarded as a function of a series of predictor variables--i.e., rate of food consumption, temperature and body weight. Sub-models also were developed for the dry matter content and energy content of fish, so that growth in energy can be converted into wet weight.

Most parameters in the model were derived from a series of controlled experiments, referred as "original experiments" in this paper. Procedures for these experiments were described elsewhere (Cui 1987; Cui and Wootton 1988a,b,c; in press a,b), and only a summary is given below. In each experiment, 25 minnows were equally divided into five ration groups--0, 1, 2 and 4% of initial body weight per day and ad libitum. Each experiment lasted 21 days, during which each fish was kept in an individual aquarium and fed daily the prescribed quantity of Enchytraeus worms. Food consumption, fecal production, excretion, dry matter content and energy content were determined directly, and metabolism was estimated indirectly as the difference between consumption and other components.

The metabolic rate of the starved fish was assumed to be the standard metabolism. SDA was assumed to be 15% of food energy, an assumption adopted in most other bioenergetics models for fish. Equations relating each of the model components were calculated from the experimental data by multiple regression. They are summarized in Table 1.

The relative dry matter content (% wet weight) and energy content (cal mg^{-1} dry matter) of the fish at the end of the original experiment were termed equilibrium dry matter content (DRYE) and equilibrium energy content (ENERGYE), respectively. The dry matter content on day t (DRY_t) was calculated as:

$$DRY_t = DRY_{t-1} + (DRYE-DRY_{t-1})/21$$

The energy content on day t ($ENERGY_t$) was calculated in a similar way. The total energy content on day t (E_t, cal) was:

$$E_t = E_{t-1}+G$$

E was converted to wet weight (W) by

$$W = (E/DRY/ENERGY)x100$$

A computer program, written in FORTRAN 77, was used to calculate the growth of fish based on a time-step of 1 day.

EVALUATION OF ASSUMPTIONS

The assumptions commonly adopted in other bioenergetics models for fish include: (1) fecal production (F) is a constant fraction of food consumption (C), (2) excretion (U) is a constant fraction of C, (3) activity metabolism

(Ra) is a fixed multiple of standard metabolism (Rs), (4) dry matter content and energy content of the fish are constant. These assumptions were not used in the model for the minnow. To assess their implications, the minnow model was modified to adopt these assumptions.

In the original experiments, the average fractions of food energy lost in feces and excreta were 0.0652 and 0.0511, respectively; the average dry matter content and energy content for all the fish used were 25.77% and 5.02 cal mg^{-1}, respectively. These values were used in the modified models.

Thus, to adopt the assumption that F is a constant fraction of C, the sub-model for fecal production (Equation 3 in Table 1) was substituted by

$$F = 0.0652C.$$

To adopt the assumption that U is a constant fraction of C, Equations 4 and 5 in Table 1 were substituted by

$$U = 0.0511C.$$

TABLE 1. EQUATIONS USED IN THE BIOENERGETICS MODEL FOR THE MINNOW[a]

No.	Equation	r^2
1	$C_{max} = 0.017W^{0.806}T^{1.22}$	0.922
2	$F = 0.032C^{0.8632}e^{0.1093T}$	0.824
3	$U = -2.0518+0.0.528C+0.2338T$, when $C>0$	0.911
4	$U = 9x10^{-5}W^{0.703}e^{0.549T-0.018T^2}$, when $C=0$	0.874
5	$SDA = 0.15C$	-
6	$R_s = 0.0162W^{0.8108}e^{0.1001T}$	0.557
7	$R_a = -24.3989+0.465C+0.017W+0.2739T^2-0.00258WT$	0.753
8	$DRYE = \exp(3.5117-0.0571\ln(FL=1)+0.0373\ln(FL+1)\ln T -0.0192\ln W\ln T)$	0.413
9	$ENERGYE = \exp(5.6722-0.4816\ln W-1.7567\ln T-0.0071Ln(FL+1)\ln W +0.2033\ln W\ln T+0.0049\ln(FL+1)\ln W\ln T)$	0.489

[a]Also shown are the coefficients of determination (r^2) of the equations fitted to the original data. C_{max}: maximum food consumption (cal d^{-1}); W: body weight (mg); T: temperature (°C); F: fecal production (cal d^{-1}); C: food consumption (cal d^{-1}); U: excretion (cal d^{-1}); SDA: specific dynamic action (cal d^{-1}); R_s: standard metabolism (cal d^{-1}); R_a: activity metabolism (cal d^{-1}); DRYE: equilibrium dry matter content (% body weight); ENERGYE: equilibrium energy content (cal mg^{-1} dry matter); FL = feeding level = $100xC_{max}$(%).

To adopt the assumption of constant dry matter and energy content, the dry matter content and energy content of fish were assumed to be constant throughout the simulation at 25.77% and 5.02 cal mg^{-1}, respectively.

To adopt the assumption that activity metabolism is a fixed multiple of standard metabolism, Equation 8 in Table 1 was substituted by

$$Ra = AxRs$$

where A = 0,1,2 or 3 and was constant in each simulation.

These modified versions of the model were used to predict the 21-day growth by a fish with an initial weight of 2500 mg at 10°C. Three ration levels were used: starvation, 3% of initial weight per day, and ad libitum. The growth predicted from each modified model was compared with that from the original model (Figure 1). The assumptions that fecal production and

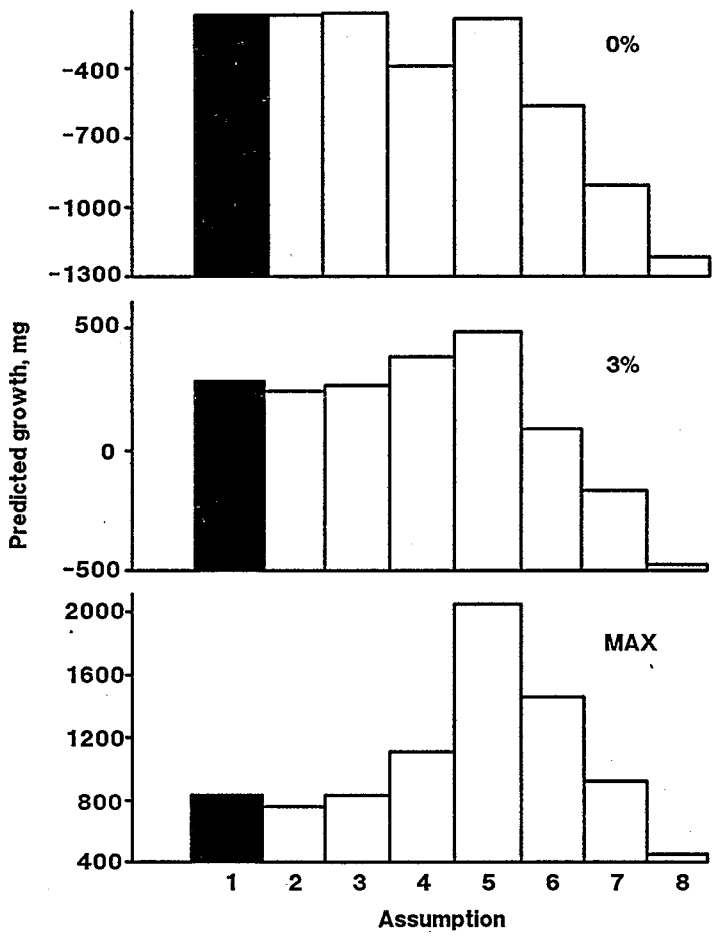

Figure 1. Effect of different assumptions on the 21-day growth of a 2500 mg minnow at 10 °C at three rations (% initial body weight day^{-1}) predicted by the bioenergetics model. Assumptions: 1. nominal model, 2. F=0.0652C, 3. U=0.0511C, 4. dry matter and energy constents are constant, 5. Ra=0, 6. Ra=Rs, 7. Ra=2Rs, 8. Ra=3Rs.

excretion are constant fractions of food energy caused only small deviations in the predicted growth; the other assumptions caused large deviations.

VALIDATION OF THE MODEL

Data from an independent experiment, referred to as "test experiment," was used to validate the bioenergetics model for the minnow. The experiment was carried out at 10°C (Cui 1987). Thirty-six minnows were equally divided into three ration groups: starvation, 3% of initial weight per day, and ad libitum. The experiment lasted 21 days. Growth, food consumption, fecal production, excretion and dry matter content of each fish were determined. The energy content was not determined but calculated from an empirical relationship between dry matter content and energy content (Cui and Wootton 1988c). Temperature, initial body weight, ration, and initial dry matter and energy content (estimated from a group of control fish sacrificed at the start of the experiment) were input into the model. The predicted growth from the model was plotted against the observed growth (Figure 2). The model gave reasonable predictions for the starved fish but over-estimated growth by the feeding fish, particularly fish fed ad libitum.

REASONS FOR MODEL FAILURE

Validation of the bioenergetics model for the minnow showed that the model was not successful in predicting the growth of fish in the "test experiment." As the model gave reasonable predictions for the starved fish, failure to predict growth at higher rations may be caused by an inadequate simulation of the growth-ration relationship. A simulated relationship between specific growth rate (SGR), conversion efficiency (K), and ration is shown in Figure 3. SGR was calculated as: $100x(\ln W_t - \ln W_o)/t$ where W_t is the predicted final, W_o is the initial weight of fish (mg), and t is the period of growth (day). K is calculated as: $100x(W_t - W_o)/C_T$ where C_t is the total amount of food consumed (mg). In the simulations, the initial weight of the fish was assumed to be 2500 mg; the initial dry matter content and energy content were 25.77% and 5.02 cal mg^{-1}, respectively; the growth period was 21 days; ration ranged from starvation to ad libitum; four temperatures were used: 5, 9, 12 and 15°C.

At all temperatures, the predicted SGR increased with increased ration. There was no approach to an asympote at high rations. The predicted conversion efficiency also increased with increased ration without a decrease at high rations. These predictions did not agree with results from most empirical studies, which showed that the relationship between SGR and ration in fish is a decelerating curve and the relationship between conversion efficiency and ration is an inverted "U"-shaped curve (Brett and Groves 1979, Cui and Wootton, in press b).

To find out why the model failed to predict the growth of fish in the test experiment, observed values for some of the energy budget components in the experimental fish were used in the model instead of the predictive sub-models. The resulting "models" were used to predict the growth of the fish and the predictions were compared with those from the original model. If use of observed values for a component yields a great improvement in the

predictions, then errors due to the sub-model for this component may be an important source for the errors in the growth predictions.

Data for the 12 fish fed ad libitum in the test experiment were used for this analysis. The total summed growth by the 12 fish was compared with that predicted by each of the modified "models" as well as the original model (Table 2). Use of observed values for fecal production and excretion resulted in little improvement in the predictions, whereas use of observed values for food consumption and dry matter and energy content yielded great improvements. This indicated that errors in growth predictions may be largely caused by the errors in the sub-models for consumption and dry matter and energy content.

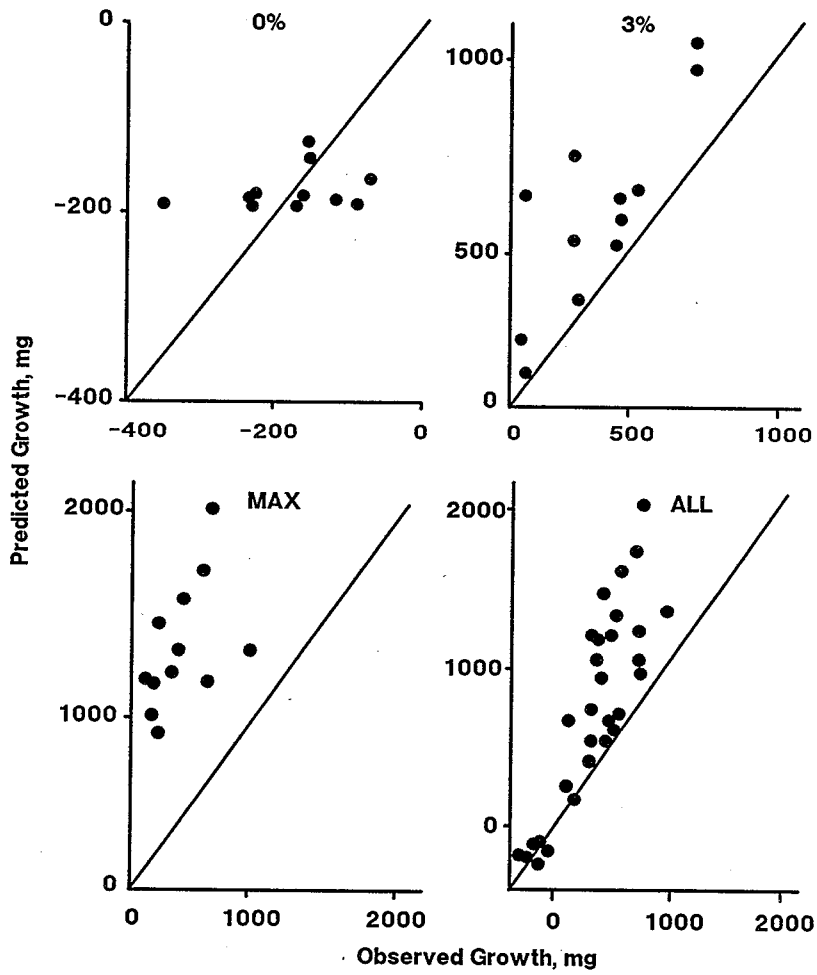

Figure 2. Relationship between the observed 21-day growth and that predicted form the bioenergetics model for the minnow in the test experiment at three rations (% body weight day^{-1}). The straight line passes through the origin and has a slope of 1.

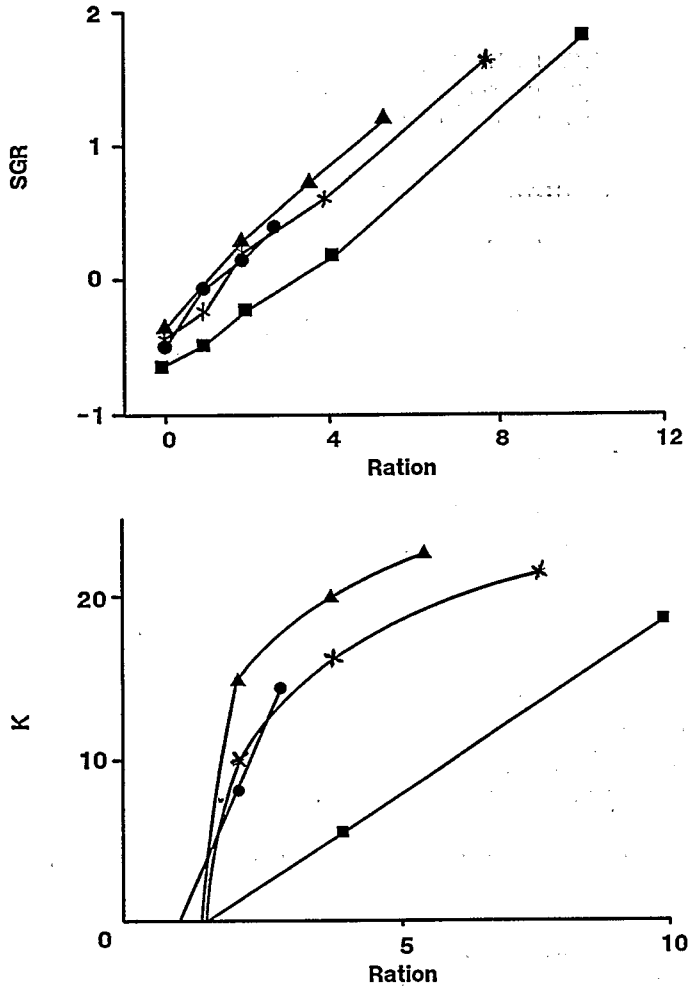

Figure 3. Relationship between specific growth rate (SGR, % day^{-1}), conversion efficiency (K, %), and ration (% body weight day^{1}) predicted by the bioenergetics model for the minnow at four temperatures--●5 °C, ▲9 °C, ✕12 °C, ■15 °C.

As food consumption is strongly related to body weight in the model, the over-estimation of consumption may be a result of the over-estimation of growth due to the poor estimates of other components. This was proved to be true as the average predicted ration, when expressed as a percentage of mean body weight, was within the range observed for the 12 fish in the test experiment. (The average predicted ration was 6.2%, while the observed values ranged from 4-7%.) When the observed values for all these components were used in the model, there was still a substantial deviation in the predicted growth, indicating that the sub-models for metabolism, which was not directly determined in the experiment, also are important in causing the prediction errors. As the model gave reasonable predictions for the starved fish, the sub-model for standard metabolism should be suitable; the errors lie in the sub-models for SDA and activity metabolism.

TABLE 2. ERRORS IN THE PREDICTIONS OF THE GROWTH OF THE 12 MINNOWS FED *ad libitum* IN THE TEST EXPERIMENT FOR "MODELS" USING OBSERVED VALUES FOR DIFFERENT COMPONENTS

		Predicted growth				
			"Model" in which observed values were used for the following component(s)[a]			
	Observed growth	Original model	C	F+U	DE	C+F+U+DE
Growth (mg)	6465	16485	13607	16792	11232	8814
Error (mg)	0	+10020	+7142	+10327	+4767	+2349
Relative error (%)	0	154.99	110.47	159.74	73.74	36.33

[a]C:food consumption, F: fecal production, U: excretion, DE: dry matter and energy content.

DISCUSSION

The results of this study indicated that, in the bioenergetics modeling of fish growth, it is relatively safe to assume that fecal production and excretion are constant fractions of food energy; however, major errors may arise from the assumptions that activity metabolism is a fixed multiple of standard metabolism and that energy content of fish is constant.

Although most parameters in the bioenergetics model for the minnow were derived from controlled experiments and the model adopted fewer assumptions that most other models of its sort, the model failed to accurately predict the growth of fish under laboratory conditions. The model's prediction of the relationship between growth and ration did not agree with that observed in empirical studies. This may be an important cause for the failure of the model.

In most other bioenergetics models, the predicted relationship between growth and ration was not explicitly examined. Kitchell et al. (1977) stated that their model for *Perca flavescens* predicted maximum conversion efficiencies at maximum rations. This was in agreement with the prediction from the minnow model.

Inaccurate estimates of dry matter and energy content, SDA and activity metabolism were the major sources of errors in the growth prediction from the minnow model. In most other bioenergetics models for fish, simplifying assumptions were made for these components.

This study suggested that, at the present stage, bioenergetics modeling may have limited use for predicting fish growth. Future studies should focus on the development of more accurate sub-models for the dry matter and energy content of fish, SDA and activity metabolism to improve the performance of the model.

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ROLE OF SCIENCE AND TECHNOLOGY IN CONSERVATION OF THE STURGEON RESOURCE

by
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INTRODUCTION

The Zhonghua sturgeon (Acipenser sinensis Gray) is an anadromous fish of enormous size, reaching a maximum body weight of 550 kg. It inhabits the continental shelf of the east coast of Asia. Every year in July and August, individuals approaching maturity ascend the Chang Jiang (the Yangtse River), where their gonads undergo further development, and spawn in the fall of the following year. Its spawning ground is distributed mainly in the lower reaches of the Jinsha River and the upper reaches of the Chang Jiang. The annual catch of the sturgeon in the provinces along the Chang Jiang averaged 400 to 500 fish in the 1970s (Yu et al. 1986).

The construction of the Gezhouba Hydro-electric Project blocked the passage for the spawning migration of this fish. The need to construct a dam by-pass for the fish was hotly debated from 1970 to 1982. Finally, on December 28, 1982, six leading officials sent a report to our central government pointing out that "construction of a fish by-pass may not be considered among the measures for the salvation of the Zhonghua sturgeon in order to avoid serious economic loss and waste," that "more impetus should be given to the experimentation on the induced spawning of the sturgeon below the dam," and that "strict prohibition of commercial sturgeon fishing must be enforced" (Academia Sinica 1984). These decisions from the central government are well grounded.

It might be helpful here to summarize the discrepancy in opinions in this matter. Those who advocated the construction of a fish by-pass maintained that the natural spawning ground of the sturgeon lies in the upper reaches of the Chang Jiang some 1000 km from Yichang. The gonads of those sturgeon detained at the Gezhouba dam could not develop to maturity, hence it would be impossible for them to spawn below the dam. For the same reason, qualified brood sturgeon for induced spawning (whose gonads must attain Stage IV development) would not be available. Therefore, the sturgeon is faced with the crisis of extinction. In their opinion, a fish by-pass is a sort of advanced installation that is indispensable. Even if the effectiveness of

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such an installation may not be satisfactory for sturgeon, it is nevertheless better to construct one than none.

Those who objected to the construction of a fish by-pass held that when the sturgeon ascend to below the dam, there was some evidence that the gonads could develop to Stage IV, at least in a part of the spawning shoal. Moreover, there are river sections downstream of the dam that offer bed conditions and hydrological features similar to those in the natural spawning ground of the Jinsha River. These sections possibly could form a new spawning ground. Experiments on the induced spawning of sturgeon in the upper reaches proved successful early in 1972. Induced spawning of the sturgeon below the Gezhouba Dam should not be "beyond the capability of our generation" as someone pessimistically predicted. What is more important is the fact that, although fish by-passes intended for sturgeons built at hydro-power stations abroad are generally unsuccessful (Doroshov 1977), the Zhonghua sturgeon has even larger body-size and sparser population, and, coupled with the vast breadth and great depth below the dam, the effectiveness of such construction seems very doubtful. Suppose we built a fish by-pass at the cost of scores of millions of yuan (RMB) that required millions of kWh of electric power to operate but achieved nothing but a "monument of stupidity" (LaBounty, J. F., personal communication).

Besides, the construction of a fish by-pass at the Gezhouba Dam is a matter that must be considered in the light of the proposed Three-Gorge High Dam. To have the sturgeon pass the high dam over 100 m above the water level is certainly much more difficult than to have it pass the low dam of Gezhouba. And if it is decided in the end that no fish by-pass should be built at the high dam, then what is the sense of constructing a fish by-pass at the dam of Gezhouba.

SCIENTIFIC AND TECHNOLOGICAL EFFORTS AFTER THE INTERCEPTION OF FLOW AT THE GEZHOUBA DAM

Interception of flow at Gezhouba was effected in January 1981. Beginning from the fall of that year, inspection of individuals with mature gonads was made each year at the sturgeon shoal at the Yichang River section below the dam. The results were 1981, 13.5%; 1982, 12.5%; 1983, 25.4%; 1984, 51.1%; (Zhao et al. 1986) 1985, 44.0%; 1986, 49.6% (Deng et al. 1987). The sturgeon aggregated below the dam normally includes individuals that will not spawn until next fall; therefore, a percentage of nearly 50% is to be regarded as normal (Zhao et al. 1986). In October-November 1982, members of the Institute of Hydrobiology disclosed for the first time a new spawning ground of the sturgeon downstream of the dam and recovered a large number of sturgeon eggs from the intestines of benthic fishes. Thirty newly hatched, live sturgeon fry also were collected 20 km below the Gezhouba Dam (Deng et al. 1987).

A joint investigation group dispatched by the National Economy Committee; Ministry of Agriculture, Animal Husbandry and Fisheries; and the Ministry of Water Conservancy and Electric Power verified this finding and wrote in their report of investigation, "The spawning and normal hatching of the sturgeon below the dam is a fact beyond any doubt and should be affirmed"

(Academia Sinica). Subsequent surveys for 3 years (from 1982 to 1984) not only confirmed the fact that the gonads of sturgeon below the dam can develop to maturity, but also delineated the site of the new spawning ground as the river section between the Yichang Shipbuilding Yard of the Bureau of Changjiang Navigation and the vicinity of Aijiahe and Jiangjunmao--a section of about 14 km in length. Besides, a brood sturgeon with running eggs was caught near Huyatan (Deng et al. 1987).

The earliest success of induced spawning of the sturgeon at the site of its natural spawning ground dated back to 1972. After the interception of flow at Gezhouba, the Collaboration Group for Induced Spawning (Liu 1987) embarked on a test for the sturgeon caught from below the dam in November 1983, adopting the method of chaining up the brood fish at the river bank. Fertilized eggs were kept in a circular "race-course," where fry were hatched. This implies the first success of induced spawning of sturgeon below the dam (Liu 1987, Fu et al. 1985). During the course of the experimentation, technical improvements were made in several ways, such as the use of the commercially available estrogen LRH-A to replace the pituitaries of sturgeon and carp completely (Yi et al. 1986), the rearing of immature female sturgeon (with ovaries of the developmental stage III) to sexually ripe parent fish in the course of 1 year (Liu 1988), and the use of a new "Sieve net" type of incubator. Newly hatched fry were kept temporarily in a loop-shaped pond, and after 7 days, they were introduced into the rearing pond at the density of 500 fry per square meter. After 137 days of rearing, the fingerlings grew to a body weight of 200 g. This result demonstrates clearly that sturgeon caught below the dam can be used for induced spawning (Liu 1987).

The Sturgeon Release Station was organized by the Fishery Division of the Gezhouba Bureau of Engineering in April 1982, and by 1984, the base was already in a workable condition. From 1982 to 1984, the station engaged in transporting the would-be spawners over the dam from below, in the hope that they might ascend further to reach the natural spawning ground. In the three fishing seasons, the station captured and released 82 fish to the newly formed reservoir above the dam (Fishery Division 1984). As to the induced spawning of sturgeon below the dam, the station obtained more than 40,000 fry from its new spawning pond and released 6000 fingerlings 4 to 5 cm in length downstream as the first batch. From 1984 to 1986, 467,000 7-day baby sturgeon 1.8 to 2.3 cm in length (69 -78 mg in weight) were released downstream (Liu 1987). Tagging of various sorts were done on fingerlings and on a limited number of large fish (average weight 715 g), but the number was too small, and the time interval too short, to get any returns as yet. A summary on the cultivation techniques of fry and fingerlings has been published recently (Xiao et al. 1988).

No matter whether natural or induced spawning is concerned, the prerequisite at present is an abundance of brood sturgeon (with ripe gonads) in the river. Regretfully, in the first 2 years after flow interception, batches and batches of brood sturgeon were captured and killed when the migrating fish were impeded by the dam. In the fall and winter of 1981, more than 800 sturgeon were caught in the Hubei Province, i.e., 5.5 times the multiyear average of 145 before dam construction. Again, a total of nearly 400 fish were caught in 1982 and in the spring of 1983 (Yu 1986). Since the

fall season of 1983, a number of Fishing Administration Stations have been set up and strict protection of the brood fish has been brought into effect. The action began to obtain good results in the second year. The number of brood sturgeon caught since 1984 has decreased to a large extent, and, in 1984, the season of natural spawning occurred earlier than in 1982 and 1983. The duration of two batches of spawning was prolonged and the range of spawning ground was extended in comparison with the previous years (Academia Sinica 1984).

Having affirmed the effects of brood fish protection, research workers now turn to the protection for the juvenile sturgeon (Zhao 1986). Their investigation in the estuary indicated that the number of specimens of juvenile sturgeon they could collect (477 in 1984 and 571 in 1985) was substantially increasing. However, juveniles descending a long way from Gezhouba were captured in large numbers by the set-net fishery at the estuary. The authors stress that the protection of juvenile sturgeon is an important link in the chain of sturgeon resource management and should arouse the attention of fishery management departments. The proposal is made to the effect that during the peak period of juvenile "emigration" (mid-June to mid-July), set-net fishing should be banned for a month, so as to give the juveniles more chance to descend to the sea for growth.

To sum up, from the refutation of the idea of building a fish by-pass for the sturgeon to the confirmation of the possibility that the sturgeon below the Gezhouba Dam can develop ripe gonads, and to the actual finding of the anticipated new spawning ground below the dam and from the improvements in the techniques of induced spawning to the protection of brood sturgeon and the installation of release stations for the sturgeon, along with the protection of juvenile sturgeon, all these point to the guiding role of science and technology in the conservation of the sturgeon resource.

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PROTECTION OF WATER QUALITY IN THE UNITED STATES

by

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Since 1970 the United States Environmental Protection Agency (EPA) has exercised primary responsibility within the Federal government to address the environmental problems confronting our nation. The Agency administers nine comprehensive environmental protection laws: the Clean Air Act; the Clean Water Act; the Safe Drinking Water Act; the Comprehensive Environmental Response, Compensation, and Liability Act (better known as "Superfund"); the Resource Conservation and Recovery Act; the Federal Insecticide, Fungicide, and Rodenticide Act; the Toxic Substances Control Act; the Marine Protection, Research, and Sanctuaries Act; and the Uranium Mill Tailings Radiation Control Act.

Some of these Acts were created since the EPA was first established; others are amended versions of legislation first enacted as far back as 1899. The 1899 legislation was the Rivers and Harbors Act, which was the first U.S. Federal law in environmental protection. Subsequent legislation in water pollution control produced the Clean Water Act, which was recently amended and reauthorized in 1987.

Using the environmental laws as the foundation, the Agency develops regulations and sets standards for pollutant levels in the individual environmental media and then monitors compliance by pollutant generators within the individual states. To ensure that its activities are based on the best available scientific and technical knowledge, the Agency conducts an extensive research and development program.

EPA is organized into four major regulatory offices to carry out its responsibilities to protect the environment and human health. These are (Figure 1) Air and Radiation, Water, Pesticides and Toxic Substances, and Solid Waste and Emergency Response. In addition, there is an Office of Research and Development with twelve research laboratories whose function is to conduct both fundamental and applied research in support of the regulatory offices. There are ten regional offices whose function is to work with the

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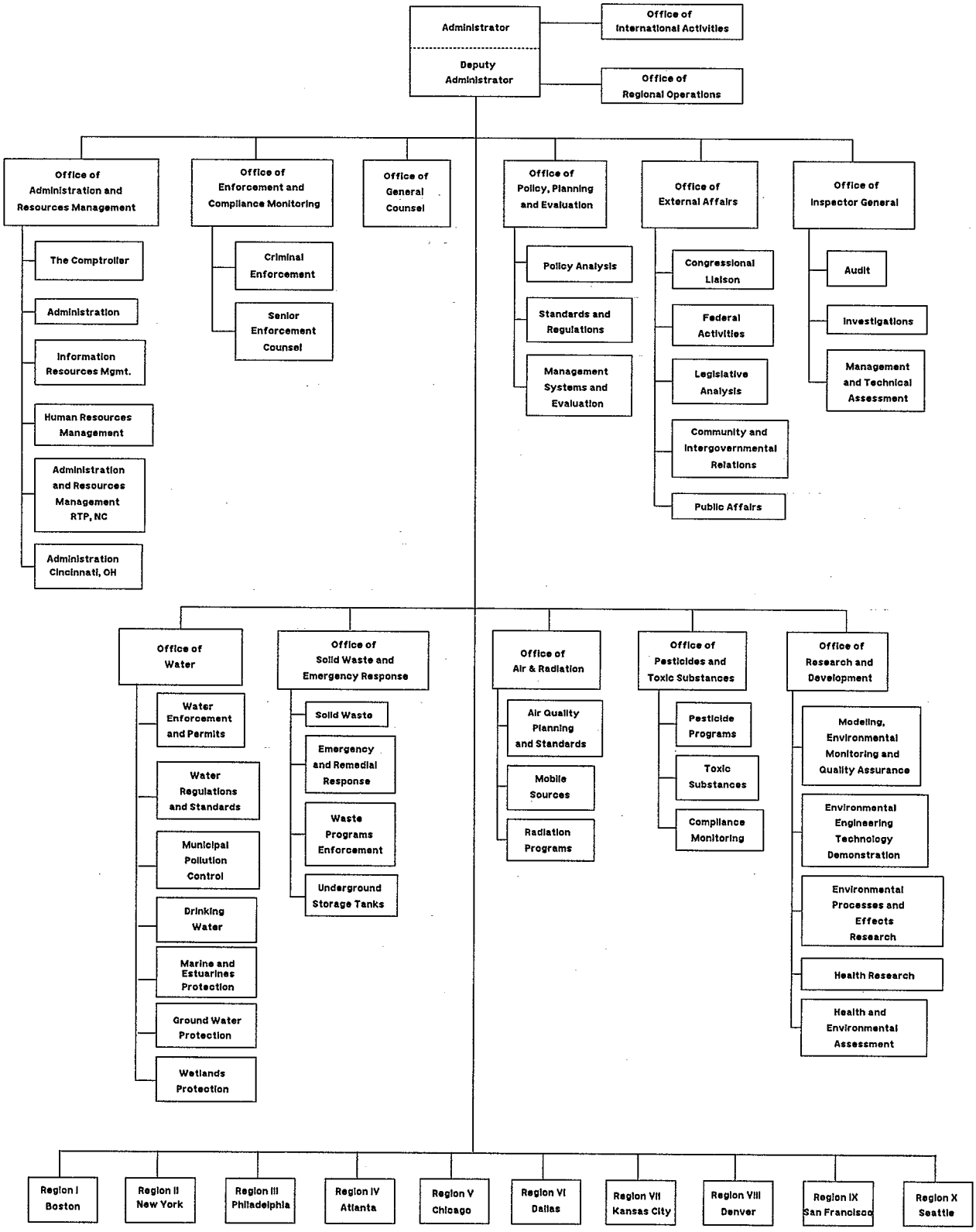

Figure 1. U.S. Environmental Protection Agency Organization

states to implement EPA's environmental regulations throughout the country. Finally, there are several administrative offices, including Administration and Resources Management; Enforcement and Compliance Monitoring; Policy, Planning, and Evaluation; International Activities; General Counsel; and Inspector General.

EPA also is empowered to enforce compliance with its regulations through administrative, civil or criminal actions in court. By seeking and winning large financial and criminal penalties against significant violators, EPA can perhaps remove any incentive to non-compliance. EPA had a strong record in enforcement in 1986 and 1987. There were 373 civil referrals in 1986, highest in history; and 274 civil referrals in 1987, second highest in history. There were 3,200 administrative orders issued by EPA in 1987, the highest in history. A record $24 million in civil penalties was assessed in 1987. Sixty percent of the total of all EPA civil penalties have been collected over the last three years.

Since 1972, EPA has awarded $44.6 billion for sewage treatment construction grants. The 1987 Clean Water Act Amendments authorize an additional $18 billion, total, for construction grants through 1990. Of this amount, at least $8.4 billion and as much as $13.2 billion is authorized for capitalization grants to establish state revolving funds. Federal money in the state revolving funds must be matched by at least 20% state funding. The State Revolving Funds will be used for construction of sewage treatment plants and other water pollution control activities. They are part of the transition from the Federal construction-grants program to a state-operated loan program. EPA is increasingly helping states develop their own criminal enforcement capabilities to detect and prosecute environmental crimes. In 1988 program enforcement priorities include an emphasis on municipal compliance for water quality. State enforcement is reaching increasingly high levels, with State agencies prosecuting a total of 723 environmental cases in 1987--twice that of the previous year. States also initiated 3200 administrative orders.

Clearly, water quality protection is a high environmental priority. The keystone for water pollution control is the National Pollutant Discharge Elimination System (NPDES). Under the NPDES, community sewage facilities must secure permits that specify the types and amounts of pollutants that may be discharged. Industries discharging pollutants into waterways also are subject to control requirements. These effluent limitations are designed to reach an ultimate goal of completely eliminating the discharge of pollutants into the nation's waters.

The water pollution control program seems to be succeeding. Since 1970, municipal sewage treatment has been provided for more than 80 million people. Most industrial plants have installed water pollution control technology. As a result, organic wastes discharged from industrial sources have been reduced by 38 percent. When all the mandated effluent guidelines are in place, discharges of toxic pollutants will have been reduced 96 percent from 1972 levels.

To deal with problems of contamination of surface waters from toxic organic pollutants such as pesticides and inorganic pollutants such as lead

and arsenic, the Agency established National Water Quality Criteria for Protection of Aquatic Organisms and Their Uses. The process of deriving national criteria is shown schematically in Figure 2 (U.S. EPA 1985). The goal of these criteria is to prevent unacceptable long-term and short-term effects on commercially and recreationally important aquatic species; other important species; fish and benthic invertebrate assemblages in rivers and streams; and fish, benthic invertebrate, and zooplankton assemblages in lakes, reservoirs, estuaries, and oceans. The national criteria for each chemical are given as two numbers: (a) criterion maximum concentration is intended to be protective against acute adverse effects due to high, short-term concentrations; (b) criterion continuous concentration is intended to be protective against unacceptable chronic effects of lower, long-term continuous concentrations.

National criteria for a specific chemical may be applied to a particular body of water directly, or may be modified to take into account local conditions of water quality and aquatic species present ("Site-Specific Water Quality Criteria"). Chemical characteristics of the local water may be such as to increase or decrease the toxicity of the chemical of concern. Some species in the local water may be very sensitive or insensitive to the chemical of concern, or the resident species may be stressed by other factors such as parasites, disease, predators, other pollutants, etc.

The states are responsible for establishing and enforcing standards for a particular surface water body. The standards are set taking into account the national criteria, any site-specific modifications of the criteria based on data about field conditions, plus social and economic considerations and environmental and analytical chemistry capabilities. EPA assists the states in setting and enforcing state water quality standards.

To regulate chemicals in surface waters, we need to know: what chemical is being discharged, the quantity being discharged, the fate of that chemical in the aquatic environment, the short- and long-term hazard it poses to aquatic life, and what the national criteria are, if available.

To derive National Water Quality Criteria, certain specific information is required: the individual chemical, data on acute and chronic toxicity to representative species of aquatic animals, toxicity to freshwater algae or vascular plants, and bioaccumulation by aquatic organisms (if a maximum permissible residue concentration in tissue is available). Other pertinent data that may be available are also taken into consideration.

Data on acute toxicity to animals consist of 96-hour LC50 (concentration causing mortality in 50% of tested animals) or EC50 (concentration causing immobility or other appropriately defined effect) values. These LC50 or EC50 values are generated by continuous exposure in laboratory toxicity tests on fishes and macroinvertebrates.

Data on chronic toxicity to aquatic animals consist of results of continuous exposure laboratory tests on fish and macroinvertebrates. These tests may use any of several endpoints to measure unacceptable effects, such as survival, growth, reproduction, or histological or other effect. Data on

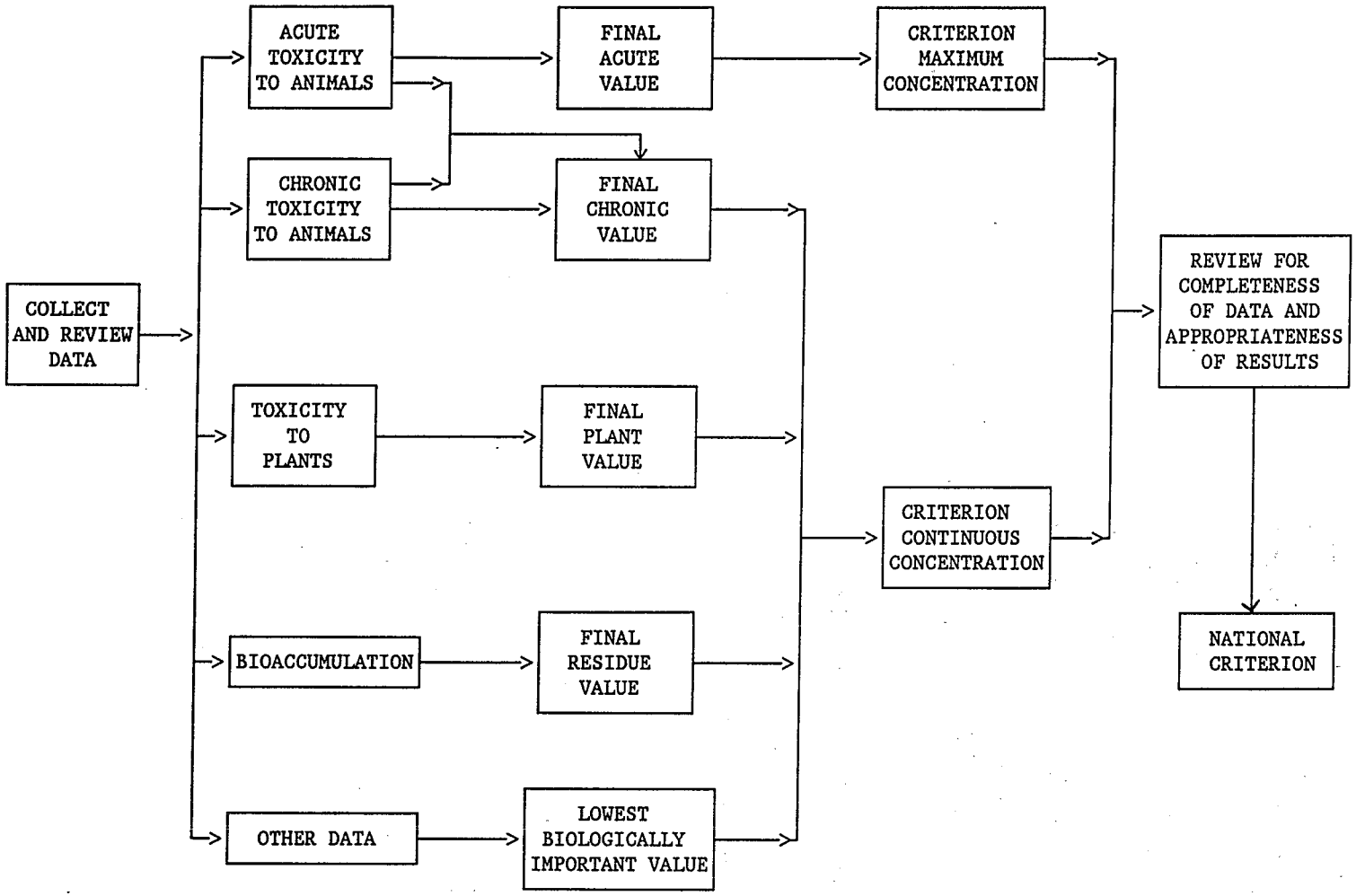

FIGURE 2. Derivation of Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses (U.S. EPA 1985).

bioaccumulation are used for chemicals where residues in aquatic organisms may be harmful to the animal; or residue concentrations may adversely affect wildlife, including fishes and birds, that consume these organisms; or residue concentrations may adversely impact human health or marketability due to flavor impairment.

For derivation of national water quality criteria (U.S. EPA 1985), the minimum set of data required for acute toxicity to aquatic animals is results of acceptable acute tests with at least one species of freshwater animals in at least eight different families such that all of the following are included.

a. the family Salmonidae in the class Osteichthyes

b. a second family in the class Osteichthyes, preferably a commercially or recreationally important warmwater species (e.g., bluegill, Lepomis macrochirus; channel catfish, Ictalurus punctatus, etc.)

c. a third family in the phylum Chordata (may be in the class Osteichthyes or may be an amphibian, etc.)

d. a planktonic crustacean (e.g., cladoceran, copepod, etc.)

e. a benthic crustacean (e.g., ostracod, isopod, amphipod, crayfish, etc.)

f. an insect (e.g., mayfly, dragonfly, damselfly, stonefly, caddisfly, mosquito, midge, etc.)

g. a family in a phylum other than Arthropoda or Chordata (e.g. Rotifera, Annelida, Mollusca, etc.)

h. a family in any order of insect or any phylum not already represented.

The minimum set of data for chronic toxicity to animals is information from tests with species of aquatic animals for which acute toxicity data are available, and the tested species must be in at least three different families. Of these three species, at least one must be a fish, at least one must be an invertebrate, and at least one must be an acutely sensitive freshwater species (the other two may be saltwater species).

Results are required from at least one acceptable test with a freshwater alga or vascular plant. Furthermore, if plants are among the aquatic organisms that are most sensitive to the pollutant, results of a test with a plant in another phylum (division) also should be available. Finally, at least one acceptable bioconcentration factor determined with an appropriate freshwater species is required, if a maximum permissible tissue concentration is available.

These data requirements were developed based on the assumption that data from toxicity tests with representative species provide a useful indication of

the sensitivities of appropriate untested species. It also must be recognized that there is a great deal of variability in toxicity testing. Species tested are often selected on the basis of availability alone; tests are conducted in laboratory waters that are free of pollutants and typically low in particulate matter and organic matter. Duration of tests can vary considerably. Also, tests on single species are used to represent biologically complex systems; i.e., to reflect the response of ecosystems to pollutants. Furthermore, these data requirements are quite expensive and time-consuming to meet. Therefore, it is worthwhile to compare the toxicity of given chemicals to a range of aquatic species. This would help to determine whether a single species might be used as a surrogate for others, and to determine what minimum amount of toxicity data could adequately reflect the susceptibilities of the aquatic animals that we are trying to protect.

Only one experimental study, by Thurston and coworkers (1985), has been conducted to determine, under standardized test conditions and procedures, the toxicity of a selected set of chemicals to a selected group of aquatic animals. The chemicals tested were selected based on their exhibiting (from literature values) a wide range of toxicity (from 26 grams/liter to 1 microgram per liter) to the fathead minnow, Pimephales promelas, and also on their having different physiological mechanisms of toxic action. The animals tested were selected based on their frequent use for laboratory toxicity tests, their ease of culture in the laboratory, and at the same time to provide a reasonable diversity of species. Six fishes, two crustaceans, a chironomid, and an amphibian were selected. Acute toxicity tests were then conducted for ten chemicals on ten aquatic species (Table 1).

Two general conclusions were drawn from this study. First, there was no consistent relative susceptibility, or orders of sensitivity, among the test species for this group of chemicals. Second, if one averaged the individual LC50 values to obtain a mean toxicity, positive deviations from the mean toxicity tended to be much greater than negative deviations. This suggests that the observed variation in toxicity between any two species for any one chemical is likely attributable to one of the species being relatively insensitive to the action of the chemical rather than one of the species being much more sensitive than the mean sensitivity for all other species.

The study also found a relationship between the toxicity data for rainbow trout (Salmo gairdneri) and fathead minnows (P. promelas), whereby toxicity data for either organism could be relied on to classify properly any of the chemicals tested. In addition, equations were developed to estimate the lethal concentrations of chemicals with each species from the toxicity data for fathead minnows.

Finally, the largest existing compilation of aquatic toxicity data, in terms of both chemicals and species, is EPA's AQUIRE (Aquatic Information Retrieval) toxicity data base (Russo and Pilli 1984), which is a computerized compilation of literature toxicity data from acute and chronic tests for freshwater and marine organisms. AQUIRE contains information from 100,000 toxicity tests for 5000 chemicals on 2300 aquatic species. Data included in

this data base are rated for quality, based on the test procedures used. Access to AQUIRE is available through the Scientific Outreach Program, EPA Environmental Research Laboratory, 6201 Congdon Boulevard, Duluth, MN 55804.

TABLE 1. COMPARATIVE TOXICITY OF TEN ORGANIC CHEMICALS TO TEN COMMON AQUATIC SPECIES (Thurston et al. 1985)

Chemical	Species
2-(2-Ethoxyethoxy)-ethanol	*Daphnia magna*
2-Methyl-2,4-pentanediol	*Tanytarsus dissimilis*
2-Methyl-1-propanol	*Orconectes immunis*
2,2,2-Trichloroethanol	*Rana catesbiana*
2,4-Pentanedione	*Salmo gairdneri*
2-Choroethanol	*Lepomis macrochirus*
Hexachloroethane	*Gambusia affinis*
Pentachlorophenol	*Ictalurus punctatus*
Permethrin	*Carassius auratus*
Endrin	*Pimephales promelas*

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THE FISH EMBRYO-LARVAL PROCEDURE: PREDICTING CHRONIC TOXICITY AND ECOLOGICAL EFFECTS

by Jeffrey A. Black and Wesley J. Birge

INTRODUCTION

Numerous studies relating to fish toxicology generally have indicated that piscine reproduction is one of the most sensitive target sites of aquatic contaminants (Birge et al. 1974). Reproductive processes can be severely impaired by toxicants at concentrations that apparently have no overt effects on adult organisms. As a result, test procedures for establishing water quality criteria have included determinations of chemical concentrations that produce chronic-level effects on aquatic life.

Methodologies historically used for such purposes have involved periods of exposure incorporating all or most life-cycle stages (Mount and Stephan 1967, Pickering 1974, McKim et al. 1976). Such tests generally require 8 months to 2 years for completion, are labor-intensive, and have impeded the generation of chronic data. To obviate such problems, more economical early-life-stage procedures were developed (U.S. EPA 1978). These tests span 30 to 90 days and chronic-effect endpoints appear to be in reasonable agreement with those observed in the longer life-cycle and partial-life-cycle tests (McKim 1977).

Because of the thousands of chemical substances requiring toxicological evaluations, however, the need persisted for still shorter and more economical tests for reliably estimating chronic toxicity to fish species. Two such "short-term" methodologies have been developed for these purposes. The first involves a 7-day test performed with larvae of the fathead minnow (*Pimephales promelas*), in which organisms are fed throughout the exposure period. Testing is initiated with 1-day-old larvae, and endpoints are quantified on the basis of survival frequencies and growth differentials between control and experimental animals. Specific procedures for the applications of this test have been described elsewhere (Horning and Weber 1985, Mount et al. 1985, Norberg and Mount 1985).

A second procedure, the short-term embryo-larval test, may be performed with a variety of fish and amphibian species. This test is initiated shortly after egg fertilization and continues through 4 days posthatching. The

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exposure period is 5 to 8 days (25°C) and typically is conducted with species such as the largemouth bass (Micropterus salmoides), bluegill sunfish (Lepomis macrochirus), and the fathead minnow. No feeding is required. Test responses include embryonic mortality, egg hatchability, larval mortality, and teratogenesis.

Log probit analysis (Finney 1971) can be used to calculate median lethal (LC50) and toxicant threshold (LC1) concentrations. These values are generally calculated by combining frequencies of dead organisms and grossly teratic larvae observed at the end of the test. Conventional statistical analyses such as Dunnett's multiple comparison procedure (Horning and Weber 1985) and the chi-square Fisher's exact test (Armitage 1971) can be used to calculate no-observed-effect concentrations (NOEC). Rainbow trout (Salmo gairdneri) also have been used in this system, in which the total exposure period is about 28 days (13°C).

These latter two tests can be performed using either static-renewal or continuous-flow procedures and have produced chronic values similar to those determined in traditional life-cycle experiments (Birge et al. 1981, Birge and Cassidy 1983, Birge et al. 1985, Norberg and Mount 1985).

This paper reviews the reliability and usefulness of the short-term fish embryo-larval test for estimating chronic-level effects of contaminant stress on aquatic organisms. Primary attention given to specific applications of the procedure, including assessments of (1) the toxicity of single compounds and chemical mixtures, (2) differential species sensitivity, (3) teratogencity as a toxicological endpoint, (4) structure-toxicity relationships, (5) effluent and receiving water toxicity, and (6) effects produced by sediment-associated chemicals.

GENERAL ASPECTS OF THE SHORT-TERM EMBRYO-LARVAL PROCEDURE

Procedures for performing the short-term embryo-larval toxicity test were reviewed briefly in the above section and have been further detailed in previous publications (Black et al. 1983, Birge et al. 1985, Horning and Weber 1985). An overview of test characteristics is given in Table 1. Selection of the particular exposure system to be used (static-renewal, flow-through) is dependent on several considerations. The static-renewal test, in which solutions are changed at regular intervals (e.g., 12 or 24 hours), is economical and can be used with toxicants that are relatively stable in solution. Flow-through procedures are recommended for testing rapidly degradable materials. One such system developed in our laboratory has been used successfully in regulating exposure concentrations of volatile or insoluble organic compounds (Birge et al. 1979a, Black et al. 1982). We presently are developing new toxicity test procedures that will require solution volumes of only 50 ml or less.

Biological endpoints subject to evaluation in embryo-larval tests are summarized in Table 2. Endpoints not discussed in this paper (e.g., avoidance/attraction behavior, tumor formation) have been described elsewhere (Black and Birge 1980, Hendricks et al. 1984). Growth, not typically used as an embryo-larval test endpoint, is currently being evaluated for reliability

Table 1. GENERAL CHARACTERISTICS OF THE SHORT-TERM EMBRYO-LARVAL TEST

1.	Five or more exposure concentrations plus control
2.	Two or four replicates/exposure
3.	20-50 organisms per 50-400 mL chamber
4.	Dilution water: natural or reconstituted
5.	Exposure through hatching plus 4 days posthatching
6.	Static-renewal or flow-through system
7.	No feeding requirement
8.	Laboratory or field applications
9.	Multiple animal species capable of being tested in one chamber
10.	Multiple test endpoints
11.	Regression analysis (LC50, LC1)
12.	Analysis of variance, Dunnett's test, Duncan's test (NOEC, LOEC)

and reproducibility. The short-term embryo-larval procedure has a number of applications and includes among others the evaluation of single toxicants, effluents and other complex mixtures, and sediment-associated chemicals. Several types of environmental media evaluated using early-life stages are given in Table 3. The following sections summarize various aspects of the test, including the reliability and applicability of the data generated.

REPRODUCIBILITY AND RELIABILITY OF EMBRYO-LARVAL PROCEDURES

The reproducibility of the short-term embryo-larval test has been investigated using various compounds, and an example of the precision of test endpoints is presented in Table 4 (Birge et al. 1985). Six continuous flow tests (two replicates each) were performed with cadmium on the fathead minnow over an 8-month period. The LC50 values varied only from 0.067 to 0.084 mg/L and LC1 values were between 0.010 and 0.014 mg/L. The 95% confidence limits were relatively small and probit-derived LC1s (threshold toxicity values) were in good agreement with NOECs determined by independent statistical procedures.

Table 2. EMBRYO-LARVAL TEST ENDPOINTS

Acute toxicity	LC50
Chronic Toxicity	Estimated chronic values or toxicity threshold values (LC1)
Teratogenesis	defective or retarded development (embryos, larvae)
Growth	length, weight
Behavior	avoidance/attraction responses, locomotor impairment, feeding
Bioconcentration	toxicant uptake

Table 3. ENVIRONMENTAL MEDIA SUBJECT TO EMBRYO-LARVAL TOXICOLOGICAL EVALUATIONS

Single chemicals/substances (soluble, insoluble, volatile)
Chemical combinations - toxic interactions
Complex mixtures
Whole effluents/dilutions
Effluent fractions
Chemical leachates
Persistence of toxicity
Receiving systems
Sediment-associated chemicals
Natural bulk sediments
Chemically enriched sediments
Sediment elutriates

Similar precision was observed in static-renewal tests, using the same compound and fish species.

The short-term procedure also has been used to perform multiple species tests, in which embryos of different species have been maintained in the same exposure chamber. In one such experiment, eggs of the carp (Cyprinus carpio), the fathead minnow, and the largemouth bass were exposed to cadmium, and the LC50 values were 138.9, 107.0, and 244.1 μg/L, respectively. The corresponding LC1s were 4.1, 11.5, and 13.9 μg/L. To achieve a broader toxicity base on cadmium with warmwater species, additional experiments were performed on the channel catfish (Ictalurus punctatus) and goldfish (Carassius auratus), and the LC1 values (μg/L) obtained with these species were 8.6 and 3.0, respectively.

The range of LC1 values calculated for these 5 species was 3.0 to 13.9 μg Cd/L. This range compared favorably to chronic values of 5.8 to 15.0 μg/L achieved in longer-term chronic tests reported in the EPA criterion document for six warmwater fish species (U.S. EPA 1980). Although data for precise species to species comparisons were not always available, these results lend support to the premise that the toxicity threshold values (LC1s) obtained in short-term embryo-larval tests can be used reliably to estimate chronic toxicity.

Further evidence of this nature has been shown in tests with the rainbow trout. Embryo-larval stages exposed in static-renewal tests to seven different metals gave LC1 values that compared favorably to maximum acceptable toxicant concentrations (MATC) calculated from chronic tests with rainbow trout and other salmonid species (Table 5, Birge et al. 1985).

TOXICOLOGICAL SCREENING OF CHEMICALS

On the basis of data indicating that the embryo-larval test can be used for predicting long-term effects, this procedure may afford a practical means

by which to screen a great number of contaminants for toxic properties, identify those of greatest concern to aquatic ecosystems, and estimate concentrations that produce chronic toxicity. As an example, the short-term procedure with the rainbow trout was tested on 33 elements found in fossil fuels (Birge et al. 1980). In this study, the LC50 values were less than 1 mg/L for 19 of 33 elements and LC1 values were 10 μg/L or less for 12. Results indicated the high sensitivity of trout developmental stages and the potential application of these procedures for use in testing programs designed to prioritize compounds for further toxicological evaluations.

The test also has been used to establish a toxicological response range for a number of different aquatic organisms. The heterogeneity of species response is important in understanding the degree to which aquatic biota vary in their sensitivity to environmental contaminants. The toxicity data on cadmium given above demonstrate the differential susceptibility among certain warmwater fish species. Another example of this concept was reflected by comparative tests in which inorganic mercury was administered to embryo-larval stages of 14 amphibian species (Birge et al. 1979b). The LC50 values spanned nearly 2 orders of magnitude, ranging from 1.3 μg/L with the narrow-mouth toad (*Gastrophryne carolinensis*) to 107.5 μg/L with the marbled salamander (*Ambystoma opacum*).

Table 4. REPRODUCIBILITY OF 8-d EMBRYO-LARVAL TESTS WITH CADMIUM USING THE FATHEAD MINNOW IN A CONTINUOUS-FLOW SYSTEM[a]

Test no.	LC50 (mg/L)	95% confidence limits	LC1 (mg/L)	95% confidence limits	NOEC (mg/L)
1	0.067	0.056-0.078	0.010	0.006-0.014	0.012
2	0.084	0.073-0.097	0.013	0.008-0.017	0.011
3	0.073	0.062-0.085	0.012	0.007-0.017	0.013
4	0.079	0.068-0.091	0.012	0.008-0.017	0.011
5	0.084	0.072-0.098	0.014	0.009-0.020	0.010
6	0.070	0.060-0.081	0.010	0.006-0.015	0.014

[a]Reprinted with permission from Environmental Toxicoloty and Chemistry, vol. 4, W.J. Birge, J.A. Black, and A.G. Westeman, Short-term fish and amphibian embryo-larval tests for determining the effects of toxicant stress on early life stages and estimating chronic values for single compounds and complex effluents, 1985, Pergamon Press.

TERATOGENICITY

Use of the short-term embryo-larval test provides the opportunity to quantify frequencies of teratogenesis present in hatched populations. For most toxicants, the number of teratic larvae generally has been found to increase with exposure level, but the dose-response relationship usually is not as precise as that observed for mortality. In most experiments, the incidence of teratogenesis has ranged from 0 to 5% at or near the toxicity threshold and frequencies of 10% or greater usually have not been observed except at or above median lethal concentrations. There are exceptions to this trend for certain metals and organic contaminants, and these occurrences have been discussed previously (Birge and Black 1977, Birge et al. 1983). Gross anomalies most often encountered have included defects of the head and

Table 5. COMPARISON OF LC1 AND MATC VALUES FOR VARIOUS METALS DETERMINED IN TOXICITY TESTS WITH TROUT[a]

Element	LC1[b] (μg/L)	95% confidence limits	MATC (μg/L)	Species	Test[c]
Cadmium	8.0	5.4-10.9	1.7-3.4	Brook trout	clc
			3.8-11.7	Brown trout	els
Chromium	21.5	10.3-35.2	51-105	Rainbow trout	els
Copper	3.4	1.6-5.9	3.0-5.0	Brook trout	els
			5.0-8.0	Brook trout	els
			9.4-17.4	Brook trout	clc
Lead	10.3	6.9-14.6	4.1-7.6	Rainbow trout	plc
			7.2-14.6	Rainbow trout	plc
			71-146	Rainbow trout	els
Mercury	0.2	0.1-0.3	0.29-0.93	Brook trout	clc
Silver	0.1	0.1-0.2	0.09-0.17	Rainbow trout	plc
Zinc	216	157-275	532-1368	Brook trout	plc

[a]LC1 values determined in 28-d static-renewal tests with rainbow trout terminated 4 d after hatching.
[b]MATCs taken from published data for 60- to 90-d early-life-stage (els), partial life-cycle (plc) or complete life-cycle (clc) tests.
[c]Reprinted with permission from Environmental Toxicoloty and Chemistry, vol. 4, W.J. Birge, J.A. Black, and A.G. Westeman, Short-term fish and amphibian embryo-larval tests for determining the effects of toxicant stress on early life stages and estimating chronic values for single compounds and complex effluents, 1985, Pergamon Press.

vertebral column, dwarfed bodies, partial twinning, microcephaly, absent or reduced eyes and fins, and amphiarthrodic jaws. Approximately 80% to 90% of the gross anomalies observed have been defects of the skeletal system. Most have involved the vertebral column and included lordosis, scoliosis, kyphosis, and rigid coiling.

Based on these observations, it was proposed that fish and amphibian embryo-larval stages may constitute simple and effective models with which to investigate teratogenesis and that such test systems possess high potential for (1) determining the mechanisms of teratogenesis, (2) evaluating the impact of environmental toxicants on aquatic biota, and (3) preliminary screening and identification of environmental teratogens that may be of concern to human health (Birge et al. 1983).

STRUCTURE-ACTIVITY RELATIONSHIPS

Because aquatic ecosystems will continuously be affected by a broad spectrum of pollutants, environmental hazard assessments must rely on an orderly and systematic process for screening toxicity of diversified contaminants. The vast number of chemicals introduced to the environment places constraints on the extent of toxicity testing that can be accomplished within a practical time frame. For this reason, considerable attention has been given to developing procedures to allow for the estimation of toxicity based on certain structural characteristics of such compounds (Konemann 1981, McCarty et al. 1985, Hodson et al. 1988). Given an adequate toxicological baseline for a representative number of organic chemicals or chemical classes, the toxicity of many untested compounds could potentially be predicted by examination of their structures or other physical-chemical characteristics.

The usefulness of the short-term embryo-larval procedure for predicting toxic effects of structurally related chemicals has been evaluated for certain classes of organic compounds (Birge and Cassidy 1983, Black et al. 1983, Millemann et al. 1984). In embryo-larval studies testing compounds within each of three chemical classes (i.e., hydroxylated aromatic hydrocarbons, azaarenes, polycyclic aromatic hydrocarbons), toxicity to both the largemouth bass and rainbow trout was found to increase with increasing ring number (Black et al. 1983). Similarly, toxicity of azaarene compounds to the leopard frog (Rana pipens) increased with ring number and increased octanol/water partition coefficients (Birge and Cassidy 1983).

Fish and amphibian short-term tests also were used to assess the relationship between toxicity and degree of chlorination of certain polychlorinated biphenyl compounds and chlorinated alkanes. Toxicity was observed to be greater as the chlorination of these compounds increased. For example, LC50 values with the leopard frog were 48, 4.16, and 1.64 mg/L for methylene chloride (2 chlorine atoms), chloroform (3 chlorine atoms), and carbon tetrachloride (4 chlorine atoms), respectively. Corresponding octanol water partition coefficients (log P) were 1.51, 2.02, and 2.79 (Birge and Cassidy 1983). Based on these and other data, the sensitive and economical short-term embryo-larval procedure may be extremely advantageous for use in broad-based testing programs designed to establish structure-toxicity relationships for a wide variety of compounds.

EMBRYO-LARVAL BIOMONITORING OF EFFLUENTS AND RECEIVING WATERS

The above descriptions of the short-term embryo-larval test primarily have been concerned with assessments of single compounds. This procedure, however, has been used successfully in a number of field investigations involving direct toxicological monitoring of effluents and receiving waters. In biomonitoring studies on effluents from a tannery-sewage treatment plant, a synthetic rubber plant, a metal plating plant, and a chemical manufacturing plant, both acute and embryo-larval tests using fathead minnows were performed concurrently on each of these NPDES (National Pollutant Discharge Elimination System) discharges (Birge and Black 1981).

The embryo-larval tests gave greater detection of effluent toxicity. The LC50 and LC1 (percent effluent by volume) values for the four effluents, as determined in flow-through tests, ranged from 0.3% to 29.4% and 0.001% to 2.8%, respectively. In the acute tests, it was not possible to calculate LC50 values for three of the four effluents. The acute LC50 for the most toxic effluent was 8%, a value about 27 times greater than the embryo-larval LC50 (0.3%) and different from the embryo-larval LC1 (0.001%) by more than three orders of magnitude.

In three of the on-site studies, tests also were conducted to determine the utility of embryo-larval biomonitoring for identifying toxic effluent fractions and for evaluating the effectiveness of waste treatment processes. Results indicated that more accurate evaluations could be achieved with embryo-larval data that reflect the "net toxicity" of complex mixtures than could be predicted with chemical analyses of the effluents or effluent fractions.

In another major field investigation, chemical monitoring, ecological surveys, hydrological measurements, and fathead minnow embryo-larval tests were conducted on an effluent from a secondary sewage treatment plant (STP) and the affected receiving stream (Birge et al. in press). A good correlation existed between embryo-larval toxicity and the degree of impact determined using faunistic survey data evaluated at the different sampling stations. In addition, an independent flow-through toxicity test performed on the STP effluent and 4 effluent dilutions gave an effluent LC1 value of 30.6% of volume. The LC1 compared closely with the instream effluent concentration (33%) observed for the last downstream monitoring station, which was the only station at which ecological survey data revealed no significant impact on fish and macroinvertebrate populations.

EVALUATION OF EFFECTS PRODUCED BY SEDIMENT-ASSOCIATED CHEMICALS

It is evident that short-term embryo-larval tests have broad uses for evaluating the toxicity of waterborne contaminants. More recently, this procedure has been adapted for determining chronic effects of sediment contamination. The importance of sediment toxicity and the development of criteria for sediment-associated chemicals are receiving considerable attention by the U.S. EPA and other interested parties. An in-depth treatment of this subject has been presented in a recent volume edited by Dickson et al. (1987).

A number of different types of sediments, both naturally contaminated and chemically enriched (spiked), have been evaluated using fish and amphibian embryo-larval stages. Test procedures and findings from these studies have been presented in earlier publications (Francis et al. 1984, Birge et al. 1987). In one such investigation, experiments were performed to determine the effects of mercury-enriched sediment on trout eggs. Samples of sandy loam sediments were taken from a local stream with biotic conditions characteristic of a healthy aquatic ecosystem and were enriched with inorganic mercury at concentrations ranging from 0.1 to 100 $\mu g/g$. Sediments were layered to a depth of 2 cm in 500 mL-Pyrex test chambers and covered with 400 mL of mercury-free reconstituted water. Eyed eggs of the rainbow trout were placed on the sediment layer and exposed from 10 days before hatching through 10 days after hatching.

A distinct inverse correlation existed between sediment mercury concentrations and percent survival (Table 6). Trout embryos and alevins accumulated significant quantities of mercury during the 20-day exposure, and tissue concentrations rose proportionally with increasing sediment levels of mercury. It is likely that contaminated sediments, when used as spawning substrates, could present a formidable hazard to fish reproduction.

With respect to these results and those reported by others (Chu-fa et al. 1979, Swartz et al. 1982, Malueg et al. 1984, and Nebeker et al. 1984), there is a considerable evidence that toxicological biomonitoring is a practical and reliable procedure by which to evaluate sediment contamination. Taking into account results based on early-life-stage toxicity tests, it is apparent that bulk sediment chemistry is important for evaluating the hazard of sediment-associated chemicals and that such tests should significantly augment the development of regulatory criteria.

SUMMARY

The embryo-larval toxicity tests described above have important implications in the assessment of chronic toxicity produced by environmental contaminants (Table 7). This procedure may be performed both in the laboratory and in the field and can be used for development of aquatic and sediment criteria, the establishment of structure-toxicity relationships, and effluent and receiving water biomonitoring. Test endpoints observed in field studies have been found to correlate closely with independent ecological parameters used to quantify effects of point-source discharges on receiving water systems. In addition, tests conducted with an effluent dilution series are useful in estimating the likelihood or degree of instream impact (Birge et al. in press). Not only is the test routinely employed for assessing chronic-level effects under the NPDES biomonitoring program, but it is also applicable for evaluating toxicity associated with hazardous waste sites in which liquid discharges, leachates, or surface water contamination are involved. Moreover, this methodology may prove to be an economical and predictive means by which to prescreen chemicals for potential effects on human health.

TABLE 6. EFFECTS OF MERCURY-ENRICHED SEDIMENT ON EARLY LIFE STAGES OF THE RAINBOW TROUT

Total mercury concentration					
Sediment		Water (μg/L)		Tissue (μg/g) Mean ± SD	Percent Survival[a]
Nominal	Actual mean ± SD	Mean ± SD	Range		
0.0 (control)	0.052 ± 0.002	0.11 ± 0.08	0.0 - 0.3	0.024 ± 0.004	94
0.1	0.180 ± 0.021	0.25 ± 0.18	0.0 - 0.5	0.036 ± 0.008	70
1.0	1.050 ± 0.043	0.15 ± 0.12	0.0 - 0.3	0.041 ± 0.001	45*
10.0	12.10 ± 1.47	1.83 ± 2.98	0.2 - 6.3	0.269 ± 0.129	23*
100.0	106.7 ± 9.9	6.40 ± 7.32	0.4 - 15.7	0.902 ± 0.059	0*

[a]Treatment was initiated at the early eyed-egg stage and continued through 10 d post-hatching, giving an exposure period of 20 d. Survival frequencies that differ significantly ($p = 0.05$) from the control value are designated with asterisks.

Table 7. APPLICATIONS OF EMBRYO-LARVAL TOXICOLOGICAL EVALUATIONS

Chemical criteria development
Ranking toxicity and animal sensitivity
Animal test species (species response range)
Chemicals (toxic effects range)
Structure-toxicity relationships
Field biomonitoring (effluents, sediments, hazardous waste sites)
Predicting ecological impact (receiving streams, sediments)
Surrogate testing for prescreening health effects (teratogenesis)

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TOXICITY OF FENVALERATE TO SIX SPECIES OF FISH AND TWO SPECIES OF FISHFOOD ORGANISMS

by

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Fenvalerate is one of the pyrethroid insecticides with broad spectrum and high activity. It is highly toxic to fish and crustaceans when it enters rivers and aquaculture ponds. Some information is needed about its toxicity to aquatic animals, especially fish that have economic value and the organisms on which they feed. In the past decade, some researchers studied the toxicity of fenvalerate to many species of aquatic animals (Mulla et al. 1978, Coats and O'Donnell-Jeffery 1979, Mcleese et al. 1980, Anderson 1982, Clark et al. 1985, Mckee and Knowles 1986, Bradbury et al. 1987). Few common fish with economic value in China and fishfood organisms were included in these studies. Only a few subacute toxicity studies of the toxicity of fenvalerate to aquatic animals have been done. The present study evaluates both the acute toxicity of fenvalerate to six species of fish with economic value in China and two species of fishfood organisms (Daphnia and algae) and the subacute toxicity on the aspects of behavioral toxicity (avoidance reaction), genetic toxicity and accumulation toxicity of fenvalerate to fish. Acute toxicity of fenvalerate to goldfish (*Carassius auratus*) and silver carp (*Hypophthalmichths molitrix*) was evaluated to provide some ecotoxicological data for the safety assessment of the pesticide.

MATERIALS AND METHODS

CHEMICALS

Fenvalerate (Technical, 90%) was supplied by the Research Institute of Hormone, Jintan, Jiangsu Province. It was dissolved in ethanol, heated slightly and then diluted to various concentrations for testing.

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STATIC ACUTE TOXICITY TEST

Test animals

Fingerlings of silver carp (Hypophthalmichths molitrix), average length 3.10 ± 0.15 cm, average weight 0.49 ± 0.15 g.

Fry of silver carp, obtained from the Aqualculture Farm of Nanjing City, 10 to 15 days old.

Fingerlings of the common carp (Cyprinus carpio), average length 3.78 ± 0.50 cm, average weight 1.06 ± 0.09 g.

Fry of the common carp, 20 days old. The common carp were obtained from the Aquaculture Farm of Nanjing.

Finless eels (Monopterus albus), average length 24.4 ± 0.9 cm, average weight 13.9 g, were obtained from the market.

Goldfish (Carassius auratus), 20 to 30 days after hatching, obtained from the market, with average length of 1.0 to 1.5 cm and average weight of 0.3 to 0.5 g.

Grass carp (Ctenopharyngodon idellus), average length 3.96 cm, average weight 1.1 g, obtained from the Aquaculture Farm of Nanjing.

The loach (Misgurnus anguillicaudatus), average length 11.9 cm, average weight 13.6 g, obtained from the market.

All the fish were laboratory-acclimated for 7 to 10 days before testing. They were not fed 24 hours preceding or during the 96-hour exposures.

Pregnant daphnia (Daphnia carinata) were cultured in 1000-ml beakers 12 hours before testing. Each beaker contained 30 animals. After culturing for 12 hours, the animals were filtered and screened with webs to obtain the newborn daphnia with instar of 12 hours.

Algae (Scenedesmus obliquus and Chlorella pyrenoidosa) were cultured with Bold Basal solution (Stein 1973) and inoculated to 1000-ml flasks containing culture media under the condition of 24 to 26°C, 2000 lux. The cultures were transferred at each 120-hour interval. After three transfers, the algae culture reached a stage of synchronus growth and they were ready for testing.

Water quality for fish test: pH 6.0 to 6.5, conductivity 220 to 310 x 10^2 μmho/cm, dissolved oxygen 7 mg l^{-1}, COD_{Mn} 1.03 to 1.19 mg l^{-1}, chloride 7.0 to 8.5 mg l^{-1}, hardness 5.38 to 6.05 (Deutsche degree).

Water quality for daphnia test: Tap water filtered by activated charcoal and aerated with micropumps, dissolved oxygen >7.6 mg l^{-1}, conductivity 1.25 To 1.60 X 10^2 μmho/cm.

Bioassay procedures: Static exposure tests using fingerling were run using 40-L aquariums, each containing 10 randomly assigned fish. A series of five exposure concentrations and a control were used. Mortality counts were recorded once every day during the 96-hour exposure. LC50 values were calculated (APHA, AWWA, WPCF, 1980). The static exposure tests using fry were run using 1000-ml and 5000-ml beakers.

A series of five exposure concentrations and a control were used for static acute toxicity tests for daphnia. Ten parallel groups were set up for each concentration. Each ten animals in one 150-ml beaker containing 100 ml of test solution were tested under the condition of 21 ± 1°C and 12 to 14-hour light periods with the intensity of 4500 to 5000 lux. Mortality counts were recorded once every day during the 48-hour exposures. The 48 hour-LC50s were calculated according to the data obtained (APHA, AWWA, WPCF, 1980).

To perform the static acute toxicity test for algae, the prepared test solution of fenvalerate was added to each 70-ml test tube containing fresh culture media for algae to make up test solutions having different concentrations, i.e., 1000 ppm, 100 ppm, 10 ppm, 1 ppm, 100 ppb, 10 ppb, and 1 ppb (6 tubes for each concentration). An ethanol control and a blank control were used.

After making cell suspensions of the algae, with cell cultures attaining the stage of synchronous growth, the cell densities were counted. The cell densities of the cultures of chlorella pyrenoidosa and Scenedesmus obliquus were 5.31×10^7 cells/ml and 7.15×10^6 cell ml, respectively. Cell suspensions of 1.13 ml and 2.09 ml were inoculated to 27 tubes containing culture medium with 3 tubes for each concentration. The initial concentrations of chlorella pyrenoidosa and Scenedesmus obliquus were 2×10^6 cells/ml and 5×10^5 cells/ml, respectively.

Culture conditions: 25 to 29°C, 2500 lux, light/dark = 14:10, shaking speed 110 rpm. The growth amounts during the culturing periods of 24, 48, 72, 96 and 120 hours were determined using a cell counting method.

ACCUMULATION TOXICITY TEST

Accumulation toxicity tests using loach (average length 11 to 13 cm, average weight 10 to 12 g) were run using 401 aquariums each containing 20 randomly assigned fish, divided into treatment groups and control. The tests were done by exposure with toxicant dose increasing progressively. The amount of toxicant in each step was 1 to 1.5 times the amount of the preceding period. The increase of doses continued until half of each group of fish were dead. The accumulation coefficients, K, were calculated by the WHO formula (WHO 1978)

$$K = \frac{LD_{50}(n)}{LD_{50}(1)}$$

where: $LD_{50}(n)$ = accumulative total dose for 50% dead animals exposed repeatedly

$LD_{50}(1)$ = dose for 50% dead animals exposed once

TEST FOR INDUCTION OF MICRONUCLEI IN THE PERIPHERAL BLOOD ERYTHROCYTE OF THE LOACH

The loaches (average length 11.9 cm, average weight 13.6 g) were laboratory acclimated for 7 to 10 days before testing. Feeding ceased before and during the exposure. The loaches were exposed both by contact with fenvalerate solution and by intraperitoneal injection. Three to five dose groups were set up according to the 96-hour LC_{50} of the loach. A positive control with mitomycin and two solvent controls with ethanol and dimethyl sulfoxide were included in the experiment. Peripheral blood was taken with a heparinized capillary tube after severance of the caudal peduncle. Blood smears were made and slides were fixed in methanol and stained with Feulgen. The erythrocytes were examined with a microscope.

AVOIDANCE TEST

Test animals: silver carp (average length 3.6 Cm, average weight 0.64 g), the common carp (average length 3.1 cm, average weight 0.9 g), and grass carp (average length 3.96 cm, average weight 1.1 g). The fish were laboratory-acclimated for 7 to 8 days before testing. Feeding ceased before exposure. Water for dilution was aerated and dechlorinated tap water. Four to six treatment groups between 1/13 to 1/2 96-hour-LC_{50} of the carp were chosen and a "clean" control and solvent (ethanol) control were set up. Avoidance reaction tests were run in straight-type avoidance equipment provided with constant water flow. Experiments were repeated four times for each concentration. Each experiment was done with ten fish, which were put previously into the avoidance test equipment for 15 minutes to acclimate. Each experiment lasted for 20 minutes. The frequencies of appearance by fish both in "clean" region and in polluted region were recorded separately each minute. Calculated data were judged by X^2 test.

TEST FOR TOXICITY OF SOAKED-OUT LIQUID THROUGH ADSORPTION BY SOIL

Test animals: fry of silver carp (10 to 15 days after hatching) were obtained from Aquaculture Farm of Nanjing City and goldfish were obtained from the market with average length 1.0 to 1.5 cm and average weight of 0.3 to 0.5 g. Soil from a rice field was collected from the suburbs of Nanjing City. The air-dried soil was put into 13 beakers (5000 ml in volume) with each 400 g. Four groups were divided according to the duration of adsorption, i.e., 24, 48, 72 and 96 hours. The concentrations for each group were 100 $\mu g\ l^{-1}$, 500 $\mu g\ l^{-1}$ and 1000 $\mu g\ l^{-1}$. A control group was included. The amount of 3500 ml of fenvalerate solution with different concentrations was poured into each beaker separately. After stirring with a glass rod, it was allowed to stand

for precipitation. The amount of 2500 ml of soaked-out liquid were drawn from 3 beakers each at the interval of 24 hours to conduct the acute toxicity test for goldfish. Ten fish were included in each experimental group. The method of toxicity test for fry of silver carp was just the same as that for goldfish but with smaller containers. The experimental temperature was 23 ± 1°C.

RESULTS AND DISCUSSION

THE ACUTE TOXICITY OF FENVALERATE TO FISH, DAPHNIA AND ALGAE

Fenvalerate is highly toxic to silver carp, common carp, goldfish and loach (Table 1). According to the classification on the degree of grade of toxicity proposed by an international group (Joint IMCO/FAO/UNESCO/WHO Group of Experts 1969), it is "very toxic" to these fish, but "toxic" to finless eel. Mcleese et al. (1980) reported that fenvalerate was highly toxic to Atlantic salmon juvenile (Salmon salar); the 96-hour LC_{50} was 1.2 $\mu g\ l^{-1}$ only. Coats and O'Donnell-Jeffery (1979) found the 24-hour LC_{50} for fingerling of rainbow trout (Salmo gairdneri) tested with fenvalerate was 76.0 ppb. The acute toxicity (96-hour LC_{50}) of fenvalerate to six estuarine fish was determined in flow-through laboratory tests by Clark et al. (1985) and 96-hour LC_{50}'s for cyprinodon variegatus, Menidia menidia, Menidia penissulae, Menidia beryllina, Leuresther tenuis, and Opsanus beta were 5.0 $\mu g\ l^{-1}$, 0.3 $\mu g\ l^{-1}$, 1.0 $\mu g\ l^{-1}$, 1.0 $\mu g\ l^{-1}$, 0.3 $\mu g\ l^{-1}$ and 2.4 $\mu g\ l^{-1}$, respectively. The values of 96-hour LC_{50}'s for five species of fish with economic value exposed to fenvalerate were coincident with these previous studies. Therefore, there is seemingly a potential menace to fishery and it is worthwhile to understand much about the toxicity of fenvalerate to aquatic animals.

TABLE 1. ACUTE TOXICITY OF FENVALERATE TO FIVE SPECIES OF FISH AND DAPHNIA

Test organism	Temp., °C	LC_{50}(ppb)			
		24 h	48 h	72 h	96 h
Silver carp (fingerling)	21 ± 1	-----	-----	3.15	2.35
Silver carp (fry	23 ± 1	-----	-----	------	8.5
Common carp (fingerling)	21 ± 1	-----	-----	------	3.8
Common carp (fry)	20 ± 1	-----	-----	------	7.9
Goldfish (fry)	23 ± 1	12.10	11.55	11.40	10.2
Finless eel	25 ± 1	-----	110.0	100.0	81.0
Loach	25 ± 1	-----	-----	------	16.9
Daphnia	21 ± 1	-----	1.14	------	-----

As one kind of fishfood organism, Daphnia do occupy an important situation in the food chain and food web in aquatic ecosystems. It is very sensitive to fenvalerate. Meyer and Ellesieck (1986) found that the acute toxicity of fenvalerate to Daphnia magna was 2.1 $\mu g\ l^{-1}$. During the 21-day test period, survival of Daphnia magna was not significantly ($p = 0.05$) affected by the 21-day exposure; however, reproduction was reduced at fenvalerate concentration of 0.25 and 0.5 $\mu g\ l^{-1}$ (McKee and Knowles 1986). The results obtained by Anderson (1982) indicated that fenvalerate can affect behavior and cause death for certain stream invertebrates at constant exposure concentration of 0.030 $\mu g\ l^{-1}$. The most sensitive animals in fenvalerate exposure were the amphipods. Within 4 to 7 days, over 50% of the gammarids at the low concentration of 0.022 $\mu g\ l^{-1}$ were dead. The results of the present study indicated that 48-hour LC_{50} for Daphnia carinata exposed to fenvalerate was 1.14 $\mu g\ l^{-1}$. We think that datum showing high sensitivity as this may be useful in the process of comprehensive analysis for the potential hazards of fenvalerate to aquatic ecosystems.

With the toxicity test for Chlorella pyrenoidosa and Scenedesmus obliquus, it was found that under low concentrations, fenvalerate showed a promotive action to these two species of algae (Tables 2 and 3). Within a certain range of fenvalerate concentrations, this action increased with increasing concentrations. The stimulating actions appeared at the concentration range of 100 ppb to 10 ppm. It is analogical to the data obtained by Chen and Zeng (1982) in their study on the toxicity of some pyrenthroids to algae. The inhibition effects of fenvalerate appeared at the higher concentration. The EC_{50} for Scenedesmus obliquus and Chlorella pyrenoidosa ranged from 100 ppm to 1000 ppm. In general, during degradation pesticides containing nitrogen and phosphorus can liberate plant nutrients. The CN^- group in fenvalerate molecule may play a role in stimulation.

RESULTS OF ACCUMULATION TEST

According to the assessment criteria for accumulation (WHO 1978), when $K \geq 5$, it belongs to "slightly accumulated." As the K value obtained by us in the present study equals 6.4, which is more than 5, there is only slight accumulation of fenvalerate in the loach. The experimental method for accumulation toxicity adopted by us is a method denoting functional accumulation. There is a relationship between the intensity of functional accumulation and the strength of metabolism as well as the degree of functional change in fish body. But information about the accumulation toxicity of fenvalerate to fish has not yet been reported. The data would be useful to evaluate the toxicity of fenvalerate.

RESULTS OF MICRONUCLEUS TEST

Results obtained are listed in Tables 5 and 6.

Results indicated that the basal value for the rate of micronucleus in the loach was rather low. It basically coincides with the data obtained by

TABLE 2. THE CHANGE OF CELL NUMBERS OF *Chlorella pyrenoidosa* UNDER DIFFERENT CONCENTRATIONS OF FENVALERATE ($x10^5$ cells/ml)

Time, hours	Number and ratio	Control (Blank)	Control (Ethanol)	1 ppb	10 ppb	100 ppb	1 ppm	10 ppm	100 ppm	1000 ppm
24**	cell number	11.70	6.40	6.13	6.13	5.87	10.10	15.50	1.07	2.93
	ratio of cell number[1]			95.8%	95.8%	91.7%	158%	242%	16.7%	45.8%
48**	cell number	20.80	2.13	1.87	2.40	1.07	1.87	2.40	1.33	1.01
	ratio of cell number			87.8%	113%	50.2%	87.8%	113%	62.4%	50.2%
72**	cell number	12.50	5.87	4.80	6.40	-----	4.00	1.33	3.20	0.80
	ratio of cell number			81.8%	109%	-----	68.1%	22.7%	54.5%	13.6%
96**	cell number	16.50	3.47	4.27	6.40	-----	8.00	4.80	2.93	1.33
	ratio of cell number			123%	184%	-----	231%	138%	84.4%	38.3%
120**	cell number	20.00	2.13	6.67	7.20	-----	4.80	2.40	2.40	0.26
	ratio of cell number			313%	338%	-----	225%	113%	113%	12.5%

** $p < 0.01$

[1]ratio of cell number $= \frac{\text{cell number of treatment group}}{\text{cell number of control group}}$

TABLE 3. THE CHANGE OF CELL NUMBERS OF *Scenedesmus obliquus* UNDER DIFFERENT CONCENTRATIONS OF FENVALERATE ($x10^5$ cells/ml)

Time, hours	Number and ratio	Control (Blank)	Control (Ethanol)	1 ppb	10 ppb	100 ppb	1 ppm	10 ppm	100 ppm	1000 ppm
24	cell number	6.93	4.27	6.67	5.07	5.87	4.53	3.47	5.07	2.13
	ratio of cell number[1]			156%	119%	137%	106%	81%	119%	49.8%
48	cell number	6.13	4.80	4.27	3.20	2.13	2.13	3.20	2.67	3.20
	ratio of cell number			89.0%	66.7%	44.4%	44.4%	66.7%	55.6%	66.7%
72*	cell number	9.07	5.33	2.93	5.07	5.60	6.67	10.90	6.40	1.33
	ratio of cell number			55.0%	95.1%	105%	125%	205%	120%	25%
96**	cell number	43.70	25.10	17.30	16.10	33.10	47.50	34.90	28.30	2.40
	ratio of cell number			68.9%	104%	132%	189%	139%	113%	9.56%
120**	cell number	28.30	19.20	25.30	38.70	42.90	48.80	40.80	22.70	2.93
	ratio of cell number			132%	202%	223%	254%	213%	118%	15.3%

* $p < 0.05$ ** $p < 0.01$

[1]ratio of cell number $= \frac{\text{cell number of treatment group}}{\text{cell number of control group}}$

TABLE 4. ACCUMULATION OF FENVALERATE

Group	Number of Fish	Dose	Number of Fish	Mortality, %	Accumulation coefficient (K)
		1/20x0.02ml	0		
		2/20x0.02ml	0		
Control (ethanol)	20	4/20x0.02ml	0	0	No accumulation
		8/20x0.02ml	0		
		16/20x0.02ml	0		
		32/20x0.02ml	0		
Treatment		1/20 LC_{50}	0		
		2/20 LC_{50}	2		
	20	4/20 LC_{50}	2		
		8/20 LC_{50}	3	50	6.4
		16/20 LC_{50}	2		
		32/20 LC_{50}	1		

Liu (1981). After exposure, micronuclei do appear, but the difference of the rates of micronucleus between treatment group and control group was not significant. It denotes that fenvalerate may not be a cytogenetic agent to fish.

RESULTS OF AVOIDANCE REACTION TEST

Results were expressed as the number of observations in clean water as a percentage of the total number of observations. Significant avoidance was judged to occur when the number of counts in the clean arm of the trough was statically higher than in the polluted arm ($X^2>3.84$, $p<0.05$) (Table 7). According to the data shown in Table 7, the concentration in which silver carp and common carp could avoid the fenvalerate were the 1/10 to 1/2 values of 96-hour LC_{50} for common carp, and that for grass carp were the 1/8 to 1/2 values of 96-hour LC_{50} ($10.3 > X^2$ 3.84, $0.05 > P > 0.01$).

The capacity of silver carp, grass carp and common carp to avoid fenvalerate solution related to the concentration of fenvalerate. The concentration threshold of fenvalerate fell in the range of 0.36 mg l^{-1} to 0.40 mg l^{-1} for silver carp and common carp and that for grass carp was 0.40

TABLE 5. INCIDENCE OF FENVALERATE TO MICRONUCLEUS OF THE PERIPHERAL NUCLEATED ERYTHROCYTES IN THE LOACH (By Contact Exposure)

Dose, ppm	Number of erythrocytes scored	Number of micronucleus	Rate of micronucleus, %	P
0.05	50000	1	0.02 ± 0.02	>0.05
0.02	30000	1	0.03 ± 0.03	>0.05
0.01	35000	1	0.028 ± 0.02	>0.05
0.005	25000	1	0.04 ± 0.04	>0.05
0.002	25000	1	0.04 ± 0.04	>0.05
Mitomycin (1.8 mg/kg)	10000	6	0.6 ± 0.24	<0.01
ethanol (0.01 ml)	50000	1	0.02 ± 0.02	>0.05
Control	60000	1	0.017	

Control: tap water

mg l^{-1} to 0.50 mg l^{-1}. The avoidance capacity of silver carp and common carp seems stronger. The avoidance capacity would be an important factor to evaluate the actual hazard of fenvalerate to fish.

RESULTS FROM TEXT FOR TOXICITY OF THE SOAKED-OUT LIQUID THROUGH ADSORPTION BY SOIL

The acute toxicity of soaked-out liquid from soil adsorption decreased rapidly along with the prolongation of time intervals for soil adsorption (Figure 1, Figure 2).

The decreasing degree was greater for those groups with higher initial concentrations. All the soaked-out liquid adsorbed by soil for 4 days did not show any acute toxicity. The decrease of acute toxicity may be due to both soil adsorption and aerobic degradation of fenvalerate (Sumito Chemical Co. 1982, Ohkawa 1980). Gan and Chen reported that through an isotope tracer experiment using ^{14}C-labeled fenvalerate they found that the mud in water system showed a strong action of adsorption and it is responsible mainly for the water purification. Fish could not only take up but also liberate the fenvalerate residues very quickly from and to waters.

To sum up, although the acute toxicity of fenvalerate to aquatic animals was very high in laboratory experiments, the actual toxicity to aquatic ecosystems in the real world would be less than predicted.

TABLE 6. INCIDENCE OF FENVALERATE TO MICRONUCLEUS OF THE PERIPHERAL NUCLEATED ERYTHROCYTES IN THE LOACH (By Intraperitoneal Injection)

Dose, ppm	Number of erythrocytes scored	Number of micronucleus	Rate of micronucleus, %	P*
0.02	20000	1	0.05 ± 0.05	>0.05
0.01	20000	1	0.05 ± 0.05	>0.05
0.005	25000	1	0.04 ± 0.04	>0.05
Mitomycin (1.8 mg/kg)	10000	6	0.6 ± 0.24	<0.01
Dimethyl Sulfoxide (0.01 ml)	20000	1	0.05 ± 0.05	>0.05
Distilled water	30000	1	0.03	

*P>0.05, not significant
P<0.01, significant

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TABLE 7. CAPACITY OF THREE SPECIES OF FISH TO SEEK WATER FREE OF FENVALERATE

Species	Concentration, μg/l	Number of fish** In water	Number of fish** In fenvalerate	Percentage in water	X^2* value
Silver carp	2.00 (1/2 96hLC$_{50}$)	22	3	87.4	13.90
	1.00 (1/4 96hLC$_{50}$)	17	6	73.0	4.66
	0.50 (1.8 96hLC$_{50}$)	15	5	74.3	4.58
	0.40 (1/10 96hLC$_{50}$)	16	7	69.5	3.89
	0.36 (1/11 96hLC$_{50}$)	13	9	59.0	0.850
	0.30 (1/13 96hLC$_{50}$)	9	7	54.6	0.101
	ethanol 0.02 ml (ml/1000 ml)	11	9	52.3	0.045
	tap water	11	10	50.0	0.046
Grass carp	2.00 (1/2 96hLC$_{50}$)	18	6	76.8	6.84
	1.00 (1/4 96hLC$_{50}$)	17	6	75.2	5.72
	0.50 (1/8 96hLC$_{50}$)	18	7	71.6	4.76
	0.40 (1/10 96hLC$_{50}$)	14	9	59.0	0.76
	ethanol 0.02 ml (ml/1000 ml)	9	8	52.8	0.056
	tap water	12	10	53.8	0.13
Common carp	2.00 (1/2 96hLC$_{50}$)	26	7	78.3	10.56
	1.00 (1/4 96hLC$_{50}$)	22	8	72.4	5.61
	0.50 (1/8 96hLC$_{50}$)	21	10	71.0	4.34
	0.40 (1/10 96hLC$_{50}$)	22	10	68.2	4.22
	0.36 (1/11 96hLC$_{50}$)	10	9	53.3	0.848
	ethanol 0.02 ml (ml/1000 ml)	16	15	51.0	0.019
	tap water	14	14	50.0	0.0005

*X^2 = P (3.84 = 0.05; 6.63 = 0.01; 10.83 = 0.001), P > 0.05 not significant; 0.05 ≥ P > 0.01 significant; P ≤ 0.01 very significant.

**Does not include fish in the holding area.

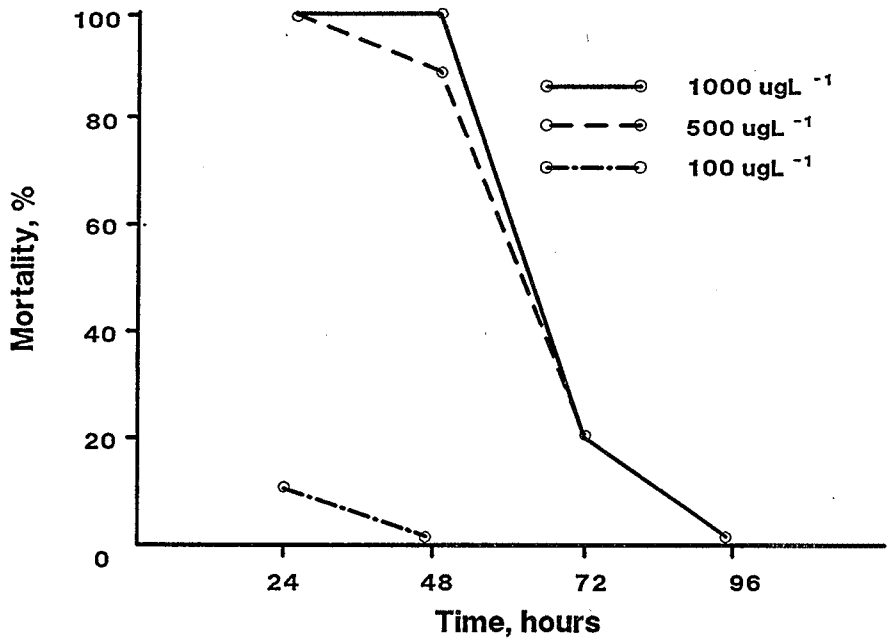

Figure 1. The acute toxicity of fenvalerate to silver carp after adsorption by soil at different intervals.

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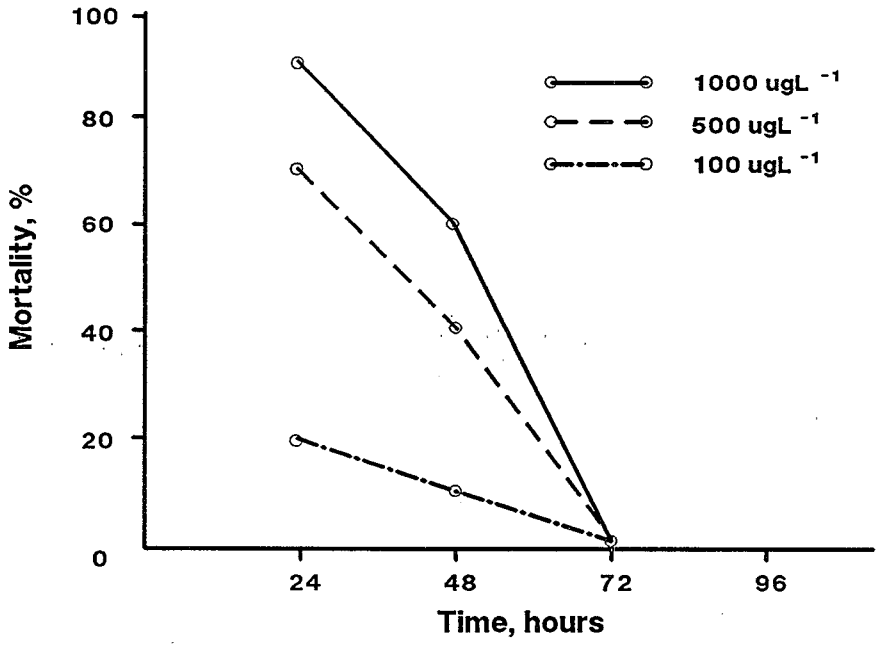

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MODELING THE EFFECTS OF TOXICANTS ON FISH POPULATIONS

by

Thomas G. Hallam[1], Ray R. Lassiter[2], Jia Li[1], and William McKinney[1]

INTRODUCTION

The relationships between toxic chemicals and their effects on populations are intricate and generally poorly understood. One reason for this lack of understanding is that the biology of the stressed organisms frequently is not considered in the determination of the risk associated with an exposure to a toxicant. The first steps in current chemical assessment procedures are generally based upon quantitative structure-activity relations (QSARs). QSARs are mathematical expressions that relate biological activity (molar concentrations causing quantal effects) to descriptors of molecular properties of a sequence of chemical compounds. These approaches are based on properties of the chemicals and do not include biological properties of the exposed organisms.

A theme of this article is that the present theoretical basis for determining effects of chemicals on populations is inadequate primarily because past developments do not account for biological detail. This inadequacy is magnified considerably when it is noted that an improper investigative focus at the population level is usually employed. These deficiencies have contributed to a lack of substantial developments in ecotoxicology. Such hindrances are especially restrictive for the foundational work relating to the determination of effects of toxicants on a biological system.

Another theme of this article is that a proper focal level for ecotoxicology must account for the individual organism. Indeed, in the classical ecological organizational scheme, the individual is special and unique. In the hierarchy from the cell to tissue to individual organism to population, the levels below the individual are sets of genetically identical elements, whereas the population is structured by genetic variation (Lomnicki 1988). It is individual variation that often is missing or suppressed in

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studies of the effects of chemicals on populations. This variability is needed to properly investigate and develop the appropriate theoretical basis for ecotoxicology. Variation in the genetical, physiological and physical distributions of individuals in a population together with the biogeochemical environment of the population determines the characteristics of the effects resulting from chemical exposure. In this article, we will generate some of the genetic and physiological distributions associated with a fish population that are relevant to the chemical effects problem.

Chemical impact occurs at the level of the individual not at the population level. Even though the target site of a chemical may be specific tissues, the exposed affected individual is the appropriate reference point for extrapolation to the population level. A basic concept of toxicology, susceptibility, intrinsically implies the existence of variance-variation that is viewed here as structure in the population. Susceptibility to chemical exposure, an individual property, is not static but is a variable related to the dynamics of the individual. Dynamic susceptibility of individuals must be reflected in the distribution of susceptibility for the population. A basic premise of this article is that effects of chemical stress at the population level are the cumulation of effects of the chemical on the individuals that compose the population.

For investigations of effects, it is essential to note distinctions between individual properties and population properties. Individual properties include physiological variables such as body size and composition as well as tolerance or susceptibility to a toxicant. Population properties include distributions of individual properties such as distribution of tolerances or susceptibility and moments of these distributions.

For modeling purposes, techniques exploring the role of the individual in determining population dynamics are a relatively recent phenomenon but are now evolving at a rapid rate (Metz and Diekmann 1986). Most of the original works in mathematical ecology unfortunately do not reflect this individual perspective, primarily because aggregation in many early studies was done at the population level. Developments at the population or higher organizational levels have proved to be generally unsuccessful in ecotoxicological studies because individual variation is lost in these representations. Recent progress in the analysis of individual-based population models is encouraging because there have been significant developments in the area of accessible computing power. This allows one to track large numbers of individuals in a reasonable computational time period. We shall utilize an approach that first develops a physiologically structured population model and then performs the analysis by numerical techniques.

This article presents a theoretical study of the effects of a lipophilic narcotic on a dynamic fish population. The work is theoretical because data, facilities and techniques are not presently available to generate the information needed for corroboration of the basic hypotheses or outcomes. The conclusions obtained are conjectures that must be tested; however, because the foundations of our model formulations are solidly grounded in the biological and toxicological literature, we believe they merit the efforts necessary to check their consistency. The effects literature is sparse because theoretical efforts in ecotoxicology have virtually ignored biology. Our efforts

represent an initial attempt to include relevant biology in an assessment procedure.

Effects are limited for the present discussion to mortality in the population. This restriction is not necessary but it is sufficient to illustrate the procedure that we suggest. The rudiments of the underlying theory for mortality in a static population are given in Lassiter and Hallam (1989a). This static theory, intuitively nicknamed "survival of the fattest," is developed for acute chemical exposures and a static population. It assesses the effects of toxic exposure by relating the n-octanol-water partition coefficient to the partition coefficient of the lipid and aqueous phases of the animal, by hypothesizing equilibration within the body, and by employing quantitative structure-activity relationships as a component of the biological response assessment. Because the chemical is lipophilic, a known distribution of lipid in the population is necessary to apply this static theory. We are aware of only two distributions of lipid in an aquatic population [Brockway (1979) and Clark (1988, personal communication)]. These distributions, for static fish populations, indicate that there can be much variation in lipid content of fish of the same relative size in both laboratory and field populations.

The dynamic behavior of individuals coupled with the possibility of chronic or multiple toxicant exposures requires a new perspective. To our knowledge, the dynamic distribution of lipid in any aquatic population is nonexistent at the present time. Nondestructive sampling methods currently are being developed and will ultimately lead to progress in this area. For our purposes, however, it is currently necessary to obtain dynamic lipid distributions by methods other than experimental ones. The approach espoused here--to focus at the biological-chemical interface of the individual--necessitates development of a dynamic representation of an individual organism. The specific individual representation employed is one developed for the purpose of determining the effects of a chemical on an individual fish. The individual model, based upon energetics and described in detail in Lassiter and Hallam (1989b), is an important part of the population model. A brief summary of the individual model is presented below so that its role in the dynamics of the population can be understood.

Exposure and uptake of chemical also must be modeled to determine the effects of a toxicant on a population. We utilize an analogue of the uptake model, FGETS, developed by Barber et al. (1988). GETS and FGETS were both formulated for fish and need little modification for our purposes.

Population dynamics are a compilation of all individual dynamics. We model population dynamics by employing a partial differential equation that incorporates individual dynamics explicitly in the population representation and describes the behavior of the population in terms of a density function that depends on individual physiological model variables and time. The population model, its behavior in the absence of the chemical, and the behavior in the presence of the toxicant will be discussed here.

The primary objectives of this paper are to indicate theoretical developments formulated to explore the effects of a chemical on a fish population when toxic exposure is allowed through both the environmental and

the food chain pathways. In a recent paper (Hallam et al. 1989), we have studied the effects of different chemicals on a *Daphnia* population. In this article, we study the effects of exposure duration on a fish population.

A MODEL OF INDIVIDUAL FISH DYNAMICS

The dynamics of an individual fish are well documented in the literature; see books in the series edited by Hoar and Randall (1969). Shul'man (1974) explores the role of lipids and their dynamics in fish populations. Other papers that explore energetics and model growth in fish population include Stewart et al. (1983) and Kitchell et al. (1974, 1977). We know of no application of energetically based models to the assessment of effects of a chemical on a fish population other than those presented here. Kooijman and Metz (1984), Kooijman (1986), and Hallam et al. (1989) have developed model formulations for *Daphnia* that are analogous to the ones presented here. Philosophically, our model is closely related to these efforts. Technically, this work is similar to our previous work on *Daphnia*; however, the specifics of the individual (and subsequently, the population model) are different than the *Daphnia* population model.

Any modeling project must be compatible with its objectives; hence, our individual model must allow for relevant interaction with the chemical and must be able to account for its toxicity. Appropriate model components must be chosen with the specific chemical and type of exposure in mind.

Most industrial chemicals and many chemicals of environmental concern are non-ionic, are nonreactive, induce baseline narcosis and are, to some degree, lipophilic (Veith et al. 1983). The chemicals employed in our illustrations are assumed to have these characteristics although the procedure does not explicitly require any of these characteristics except the lipophilicity and the nonreactivity. A method of determining the toxicity effects threshold such as application of a QSAR also is needed. The lipid in an individual buffers the action of a lipophilic chemical, allowing larger body burdens in fatter individuals than in less fat organisms to elicit biological response. Thus, additional lipid in an individual results in an extension of the toxicity effect thresholds for an acute exposure. Lipid storage provides protection against toxic stress from transient exposures only if the organism is not forced to rapidly mobilize quantities of stored lipids. When an organism rapidly utilizes stores of lipid in a situation where high body burdens have been obtained, internal release of the chemical can lead to toxicity effects under conditions where there is no change in the external environmental concentration of a chemical. These considerations indicate that, for the class of chemicals under consideration, a lipid compartment is important and appears necessary in any individual model that is utilized to represent the biological-chemical interaction.

A MATHEMATICAL MODEL OF A FISH

We now summarize the model of the life history of a fish. More detail and background information can be found in Lassiter and Hallam (1989b). Figure 1 is the conceptual model listing associated compartments and the flow

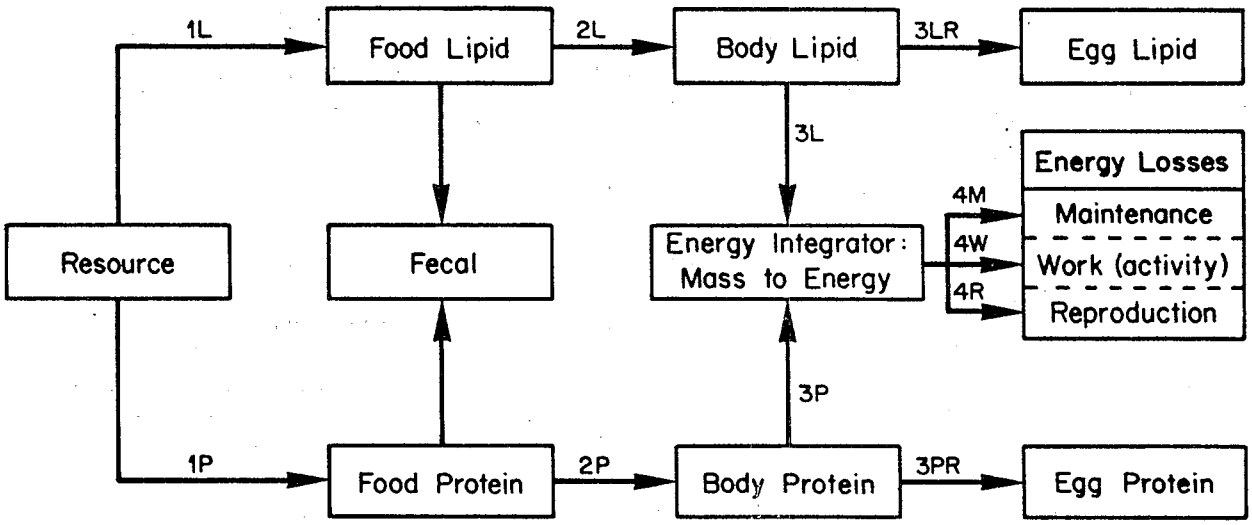


Figure 1. Compartment and flow diagram for the individual fish model.

chart for an adult female fish. The model assumes that the only inputs to the lipid and protein compartments are obtained from a decoupling of the food lipid and structure; that is, no synthesis of fat can occur from the carbohydrates and proteins of the resource. This is not a valid hypothesis in most situations (Vel'tishcheva 1961, Amlacher 1961, Menzel 1960) as Shul'man (1974) indicates. On the other hand, the diets of many fishes contain small amounts of carbohydrates that can be converted into lipids, so in these cases this model might be useful.

Let m_L and m_S denote the mass of the lipid and mass of the structure, respectively, in an individual organism. Structure is regarded as primarily protein and carbohydrates. Each of these components are assumed to have both labile and nonlabile portions. The nonlabile portion of the structure is viewed as protein and carbohydrates bound in soma and is designated in the model by m_{PS}, the mass of the protected structure. The labile portion of the structure component is represented by $m_S - m_{PS}$. The nonlabile lipid is assumed to be proportional to m_{PS} and in the model is represented by ϵm_{PS}; hence, the labile lipid is $m_L - \epsilon m_{PS}$.

The density of the resource is denoted by x and we assume that $x = x_L + x_S$ where x_L and x_S are the lipid and structural portions of the resource density, respectively. The resource is assumed to be utilized for growth according to a hyperbolic uptake law (Lassiter 1986). For details on the particulars of the representation used here refer to Lassiter and Hallam (1989b). The losses of energy for the maintenance and the activities compartments are assumed to operate on a continuous time scale. Hence, on intervals where there is no reproductive loss the fish is assumed modeled by the differential equations:

$$\frac{dm_L}{dt} = g_L = \frac{x_L}{[a_d M_p]^{-1} + [\frac{S_d}{M_p \delta v} + [kM_3]^{-1}]x} - \begin{cases} A_3(m_L - \epsilon m_{PS}) & TED > AE \\ A_3(m_L - \epsilon m_{PS})\frac{TED}{AE} & TED \leq AE \end{cases} \quad (1)$$

$$\frac{dm_S}{dt} = g_S = \frac{x_S}{[a_d M_p]^{-1} + [\frac{S_d}{M_p \delta v} + [kM_3]^{-1}]x} - \begin{cases} A_4(m_L - \epsilon m_{PS}) & TED > AE \\ A_4(m_L - \epsilon m_{PS})\frac{TED}{AE} & TED \leq AE \end{cases}$$

The quantity a_d is the encounter rate coefficient (liters per day) obtained from Gerritsen and Strickler (1977) and is given by

$$a_d = 8.64 \cdot 10^4 \pi s^2_d (v^2_p + 3v^2_h)/10^3 3v_h \quad (2)$$

with s_d defined as the reactive distance (cm) of the fish (Breck and Gitter 1983) and given by $S_d = 4.775 L_f^{1/2}$, where L_f denotes the length of the fish; v_p is the velocity of the prey (cm/s) and is given by $v_p = blsp \cdot L_p$ where L_p denotes the length of the prey and blsp denotes the body lengths per second of the prey; and v_h is the velocity of the fish (cm/s) while hunting for prey defined by (blsh) (Lf) with blsh denoting the body lengths per second of the fish while hunting. The symbol M_p represents the mass of the prey (g dry wt); $\underline{\delta}v$ is the difference in the swimming velocities of the fish and its prey: $\underline{\delta}v = (v_c - v_p)8.64.10^4$; k is the gut emptying rate ($1/d$) and M_s is the mass capacity of the fish's stomach (g).

A generic derivation for the feeding rate of a pursuit feeder may be found in Lassiter (1986, Equation 66). The formulation is a hyperbolic function $F = x/(A_1 + A_2 x)$ in g/d where x is the density of the prey population. When formulated in terms of mass consumed, the above form may be written as a hyperbolic function of numbers of prey N_p:

$$F = \frac{x}{[a_d N_p M_p]^{-1} + [\frac{S_d}{M_p \delta v} + [kM_s]^{-1}]x}$$

The terms in the denominator relate to characteristic times (d/g) for pursuit, encountering prey, and digestion, respectively:

$T_c = S_d/(\delta v M_p)$ (d/g captured),

$T_e = 1/(a_d N_p M_p)$ (d/g captured),

$T_d = 1/(kM_s)$ (d/g eliminated).

The feeding rate is thus $F = (T_c + T_e + T_d)^{-1}$. In our computations, we assume that the number of prey is fixed. The reproductive losses are assessed at discrete times of reproduction. Reproduction is allowed in a time window to represent seasonality aspects of reproduction. This reproduction interval is utilized to set the birthing times for the specific species and is utilized because we do not indicate hormone triggers such as temperature-related phenomena. The losses associated with the individual fish model include energetic needs associated with maintenance, activity and reproduction as well as losses of lipid and structure for egg production. Maintenance energy requirements are assumed to be proportional to the compartment. Activities are modeled according to Gerritsen (1984) who states a relationship for power consumed by swimming fish as

$$P = 0.275Av^{2.5}L^{-0.5}q^{-1} \qquad (ergsec^{-1}g^{-1})$$

where A is wetted surface area ($0.4L^2$), v is swimming velocity (part of the time hunting, part of the time chasing and is given as the appropriate product of bls and L), and q is swimming efficiency (0.2 according to Gerritsen). Thus, after converting to appropriate power units

$$P = 4.75.10^{-3}bls^{5/2}L^4$$

or when cruising and chasing are included

$$P = 4.75.10^{-3}L^4M_f(blsh^{5/2}T_eF+blsc^{5/2}T_cF)$$

The reproductive losses are assessed at the discrete times of reproduction. It is clear that the process of allocation of bulk mass to eggs occurs over a continuous time frame but, because there is little information on the specific time scales of this process and because it is small relative to population time scales, we treat them as discrete events. The reproductive losses include bulk allocation to eggs and the energy required to deposit this mass in the eggs. These operations, as well as the mechanism employed to determine the number of eggs produced, are described in detail in Lassiter and Hallam (1989b) and Hallam et al. (1989).

Examples of the numerical solution of the individual model are given in Figure 2.

UPTAKE IN AQUATIC ANIMALS

The uptake model that is employed in connection with the above individual model is a modification of the FGETS model (Barber et al. 1988). The model, based upon thermodynamic potential, represents the chemical exchange between a fish and the aqueous environment that occurs across gill membranes and the chemical exchange that occurs across gut walls and the contaminated ingested

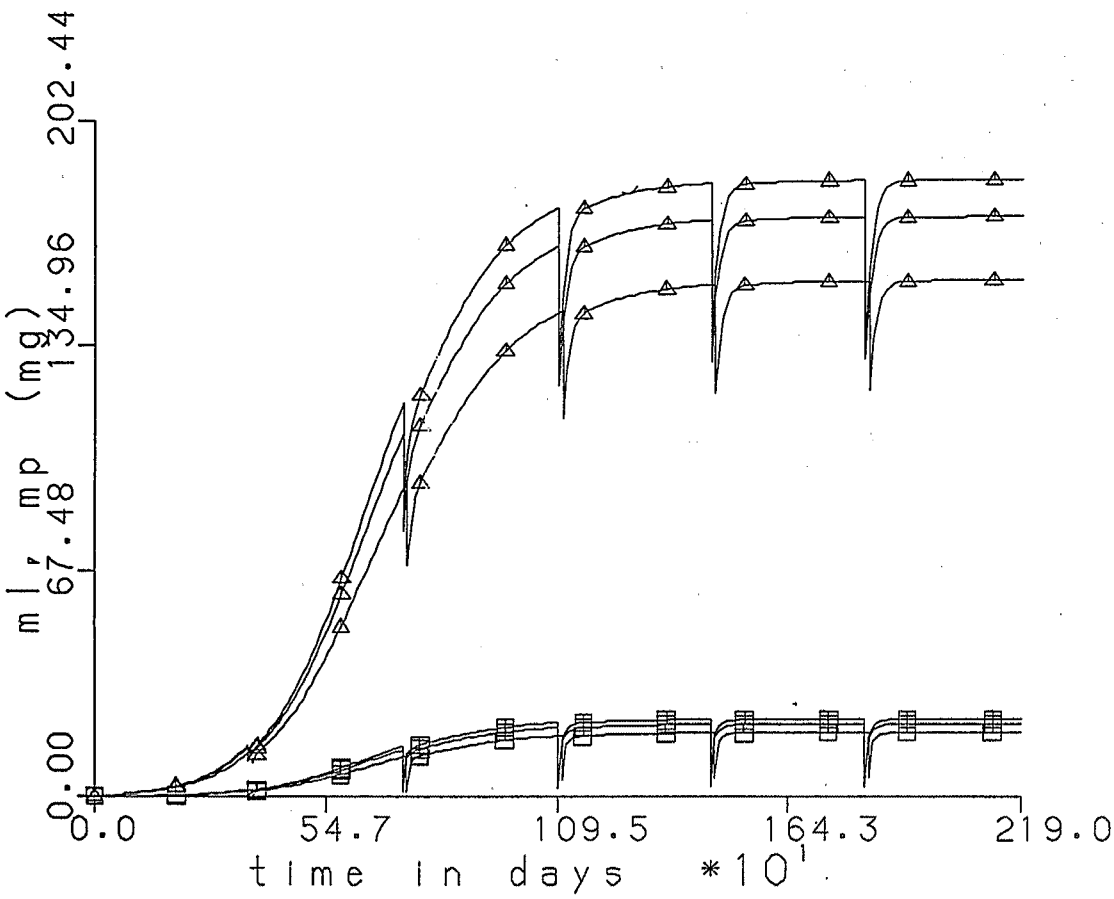


Figure 2. Lipid (m_L) and structure (m_S) cycles in the individual model. The dynamics illustrate the decrease of size while in the egg sack, exponential growth as a juvenile, and the reproductive cycles. The nonlabile structure, mps, is a nondecreasing function of age.

food. The model in its simplest form without food uptake is of the classical bioaccumulation form; however, in this approach the parameters of the uptake model are represented in considerable detail and include representations of many factors that can influence internal concentrations such as the fractions of the organism that are lipid (P_L), aqueous (P_A) and structure (P_S); the partition coefficients that indicate the affinity of the chemical towards lipid (K_L) and structure (K_S); the conductance of the exposed membrane (K_g); the total weight of the organism (W_T) and the active (effective) exposure area (S_g) of the gill. The general form of the model is thus that of a classical bioaccumulation model plus a term for exchange across the gut:

$$\frac{dB_T}{dt} = S_g k_g C_W + C_F F - B_T \left(\frac{S_g k_g}{W_T \cdot BCF} + \frac{E k_E D_A}{W_T P_A} \right) . \qquad (3)$$

In Equation 3, B_T represents the total toxicant in the organism; C_W and C_F represent the concentrations of toxicant in the environment and in the

food, respectively; F and E are the mass fluxes of food and feces, respectively; BCF is the bioconcentration factor (total concentration in the organism/C_W); k_E is the partition coefficient of chemical to excrement; $C_E = k_E C_A, C_A$ is the concentration of toxicant in the aqueous portion of the organism; and D_A is the aqueous distribution coefficient ($D_A=(1+P_S P_A^{-1} K_S+P_L P_A^{-1} K_L)^{-1}$. The unit conductance k_g may be calculated from the molecular weight of the chemical and the n-octanol-water partition coefficient. Barber et al. (1988) show that k_g is approximately $N_{Sh} D_W h_W^{-1}$ where N_{Sh} is the Sherwood number, D_W is the toxicant's diffusion coefficient, which is a function of the molecular weight of the chemical, and h_W is the characteristic dimension of interlamellar channels.

A seemingly natural approach to model the uptake of chemical from the food chain pathway would be to proceed by employing hypotheses similar to those imposed for the environmental pathway. The complications of this hypothesis and other options for modeling the uptake from contaminated food are discussed in Barber et al. (1988).

The basic assumption of this model representation, that of equilibration of chemical between the organism's body and the gut contents, is, of course, not necessarily true. It has been demonstrated that this requirement is a worst case assumption during increasing body concentration when exposed to contaminated food; that is, no additional chemical could be taken up under any thermodynamically consistent assumption than would be taken up when food and body equilibrate. During depuration, however, this assumption leads to predicted minimum depuration times; that is, any other thermodynamically consistent assumption would lead to longer depuration times. For toxicity evaluations, this would usually not be considered the worst case scenario.

EFFECTS OF TOXICANTS ON INDIVIDUALS

The basic ideas employed to assess the effects of chemicals on an individual and on a static population are given in Lassiter and Hallam (1989a). We review these ideas to set the stage for this study on the effects of a chemical on a population. Again the particular effect focus is on mortality of the individual but sublethal effects could be considered by the same methods.

The assessment of mortality due to chemical action is implemented by utilizing formulations obtained from QSARs. The processes relating to mode of action and concentration-response relationships must be coupled to determine the effects. The procedure then consists of combining the following into an assessment protocol:

(a) The differential equations for development of the individual dynamics.

(b) The differential equation for the total concentration of toxicant (or, equivalently, the total body burden) in the organism.

(c) The determination of mortality from QSARs.

The first two of these steps couple the dynamics of the individual with the dynamics of the chemical in the individual. The chemical uptake is coupled to the individual through the weight terms in each of the Equations 1 and 2. Step c, the assessment of mortality, utilizes results of Veith et al. (1983) and Konemann (1983) (see Figure 3). These bioassays are developed for baseline narcotic chemicals and relate a chemical property, K_{OW}, to mortality of the individual.

There are numerous QSARs in the literature for modes of action other than baseline narcosis. For example, the modes of polar narcosis (Veith et al. 1985) and uncoupling of oxidative phosphorylation (Schultz et al. 1986) have been documented. A common feature of each of these modes of action is that lipophilicity of the chemical as measured by K_{OW} is important. We do not present modes of action other than narcosis. Because the analogous QSARs are based at least in part upon K_{OW}, however, it appears that assessment of risk due to exposure to a chemical with these other modes also must utilize lipid as an individual model component.

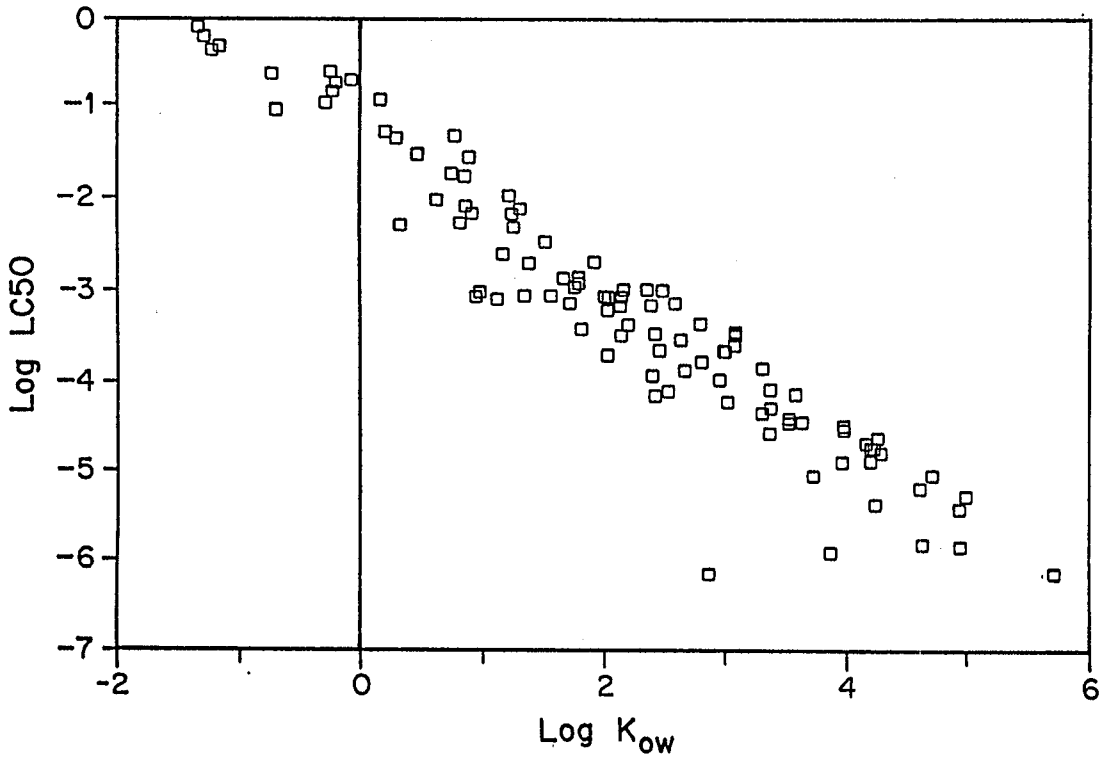


Figure 3. Relationships between log LC_{50} and log K_{OW}. Fitted result is log (LC_{50})=-0.8 - log (K_{OW}). Data from Veith (1983) and Koneman (1981).

AN ACUTE EXPOSURE-STATIC POPULATION THEORY: SURVIVAL OF THE FATTEST

In a recent article (Lassiter and Hallam 1989a), we have developed an approach that utilizes individual variation to structure a population and to explain the effects of an acute toxicant exposure from a lipophilic narcotic on that population. The basic idea is that lipid provides a buffer against toxic stress and this factor must be utilized in any consideration of effects. According to this theory, in an assessment of mortality, an individual with a smaller lipid fraction body content will die before another individual with a larger lipid fraction given equal exposure (Lassiter and Hallam 1989a). The hypotheses for this theory are related directly to the static state of the population. This static state allows only acute toxicant exposures. The pathway of exposure is not important for effects on static populations.

A DYNAMICS THEORY: EFFECTS OF TOXICANTS ON POPULATIONS

Our prototype dynamic population model is based on individual response so that effects can be determined directly and the cumulative effect at the population level ascertained. The toxicant-population model is formulated so that a toxicant may be released at numerous times and for arbitrary exposure lengths during the study period so that chronic as well as acute exposures may be investigated.

First, we will sketch the approach used to model the population. Then, we will discuss the effects of the toxicant on the population due to duration of exposure.

An approach that allows incorporation of individual dynamics into a dynamic population formulation is the McKendrick-von Foerster equation (Metz and Diekmann 1986). This partial differential equation allows explicit representation of physiological variables as they determine the dynamics of individuals. It also keeps track of the total population through the population density function. The assessment of effects of a chemical that is a lipophilic narcotic mandates that the individual model should minimally include some measures of the physiological variables age, lipid, and structure. Age is necessary to reflect the life history of an individual. Lipid, a bioconcentration site for the toxicant, is necessary to assess the effects of the chemical on the individual. Structure is necessary to account for size related measurements such as weight or length of the organism.

If $\rho=\rho(t,a,m_L,m_S)$ is the population density function--which depends upon time and the physiological variables a, representing age; m_L, representing the mass of the lipid, and m_S, representing the mass of the structure compartment--and g_L and g_S are the growth rate of the lipid and structural components of an individual as given by the Equations 1 and 2 respectively, then an equation that incorporates these physiological variables into a population scheme is

$$\rho_t + \rho_a + (\rho \cdot g_L)m_L + (\rho \cdot g_S)m_S = -\mu\rho$$

The subscripts denote partial derivatives with respect to that subscript.

The birth process for the population is represented by a boundary condition, and the mortality rate is given explicitly in the differential equation as μ. For physiologically structured models, the particular form of the birth process may be written in several equivalent representations. One of these forms is

$$\rho(t,0,m_{L_0},m_{S_0}) = \iiint \beta(t,a,m_{L_0},m_{S_0},m_L,m_S)\rho(t,a,m_L,m_S)\,da\,dm_L\,dm_S,$$

where m_{Lo} is the mass of the lipid and m_{So} is the mass of the structure at age=0 and β is the birth function which represents the number of eggs with lipid content m_{Lo}, structure content m_{So} born to an individual of age a with lipid content m_L and structure content m_S at time t. One can use this form because there is a one-to-one correspondence between lipid or structure mass and age for fixed m_{Lo}.

Several different types of mortality are represented in our numerical model formulation. We include formulations for age dependent mortality, size dependent mortality, and density dependent mortality. The age dependent mortality is assessed uniformly along cohorts, whereas the density dependent mortality is assessed uniformly across the population. The size dependent mortality is viewed as possibly caused by predation and is determined by weight of the individual. The population model is, in general, nonlinear but it would be linear if the density dependent mortality term were omitted. Some restriction on mortality generally is needed to prevent the population from growing beyond reasonable size. The specific forms of these mortalities are generic and have a form that can be structured to test processes. Formulations of the mortalities are given in Figure 4.

This population model is a first order hyperbolic partial differential equation that may be represented in an alternate manner by the method of characteristics. In this method, the partial differential equation is reduced to a set of ordinary differential equations that are valid along certain special curves called characteristics. Specifically, in this model an equivalent representation for the partial differential equation is the following system of five ordinary differential equations.

$$\frac{da}{d\lambda} = 1$$

$$\frac{dt}{d\lambda} = 1$$

Individual Mortality:

Weight–Dependent Mortality : $\mu_W = \gamma_W \cdot \mu_{1W} = \gamma_W \cdot \mu_{1W}(W), \quad W = \text{weight (mg)}$

$$\mu_{1W} = \begin{cases} V_0, & W = 0 \\ V_C, & W \in [W_1, W_2] \\ V_F, & W = W_3 \\ \text{continuous and linear}, & \text{elsewhere} \end{cases}$$

Age–Dependent Mortality :

$$\mu_A = \begin{cases} \kappa a, & 0 \le a \le 50 \\ \infty, & a > 50 \end{cases}$$

Population Mortality:

Density–Dependent Mortality : $\mu_D = \mu_D(P_B), P_B =$ total population biomass.

$$\mu_D = \begin{cases} D_M, & P_B \in [0, P_T] \\ D_0, & P_B = P_0 \\ 2D_M, & P_B \ge P_C \\ \text{continuous and linear}, & \text{elsewhere} \end{cases}$$

Figure 4. Mortality functions used in population model.

$$\frac{dm_L}{d\lambda} = g_L$$

$$\frac{dm_S}{d\lambda} = g_S$$

$$\frac{d\rho}{d\lambda} = -(\mu + (g_L)_{m_L} + (g_S)_{m_S})\rho .$$

Our approach is to solve a transformation of this system of codes numerically. Examples of graphical representations of these numerical solutions are given later for several situations of interest.

The behavior of the population in the absence of a toxicant is oscillatory on several scales. There are oscillations on the time scale of the

(assumed periodic) reproductive time (gestation period) of the species. There are longer term oscillations that do not seem to be related to mortality but rather to the rate at which the organisms grow. This longer term oscillation is not apparently due to the dynamics of the resource, which is assumed to be at constant density. In particular, this is not a typical predator-prey oscillation where both predator and prey have oscillatory behavior. However, this oscillatory behavior of a consumer when the resource is at a relatively constant level is characteristic of some aquatic populations (Murdock and McCauley 1985) that do oscillate in the presence of a nonoscillatory resource. The cause of the longer term fluctuations is apparently not a result of the assumed density-dependent regulation. We mention this because the folklore in rudimentary nonstructured models indicates that oscillations are often caused by inclusion of density dependence (Hallam 1986).

Our numerical procedure essentially follows cohorts of individuals along characteristics. This allows effects of toxicant exposures to be assessed at the individual level, a desirable attribute as we have indicated above, but the overall effects on the population can still be determined by an accumulation of individual effects. The addition of a toxicant leads to another type of mortality assessment. This mortality due to toxicant exposure is assessed at the individual level according to lipid content of the individual. Hence, in the model, there are four essentially distinct causes of death: mortality due to the physiological process of aging, mortality due to size (as, for example, determined by predation), mortality due to density dependence (as, for example, determined by total population size), and mortality due to toxicity (as, for example, determined by the lipid distribution in the population).

The population structure and analysis as reported here is based upon 27 different types of individuals. These individual ecotypes (morphs) are determined by the constant level of resource at which they feed, the quality of that resource as indicated by its lipid content, and the gut clearance rate of the organism. Each of these three individual characteristics ranges through three levels for the total of 27. Any number of individuals can be employed in the model. We employ 27 because the diversity generated by the 27 ecotypes is considerable. Figures 5 and 6 indicate the graphs of several of the ecotypes of individuals. The parameters we employ are selected from the literature on trout although there are several requiring estimation (Lassiter and Hallam, 1989b).

The population model records the dynamics of cohorts of individuals in the population, assesses mortality in these cohorts, and indicates births. Organisms are assumed to be clones of their female parent; this may not be a proper hypothesis for fish, which are not parthenogenetic.

Our objectives are to indicate the processes that affect studies on effects of toxicants on populations and, although the importance of many environmental parameters on ecological systems is recognized, we have not included important relationships such as seasonality, temperature, and pH in process formulations. In general, these environmental variables affect only the parameter values in our process representations and not the formulations themselves.

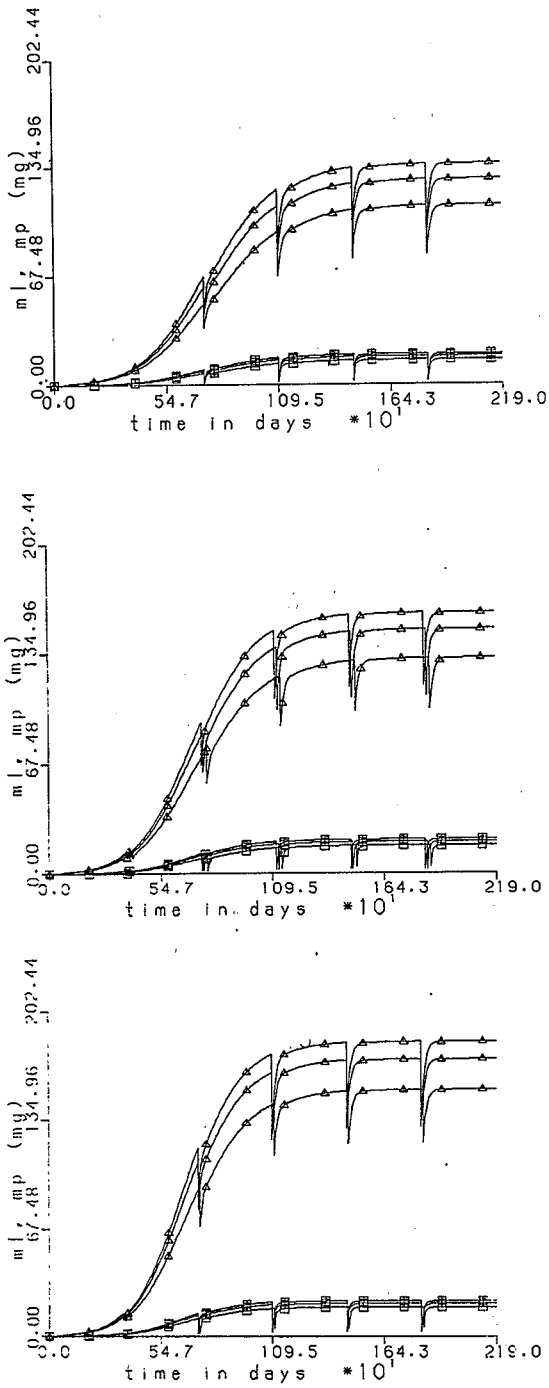
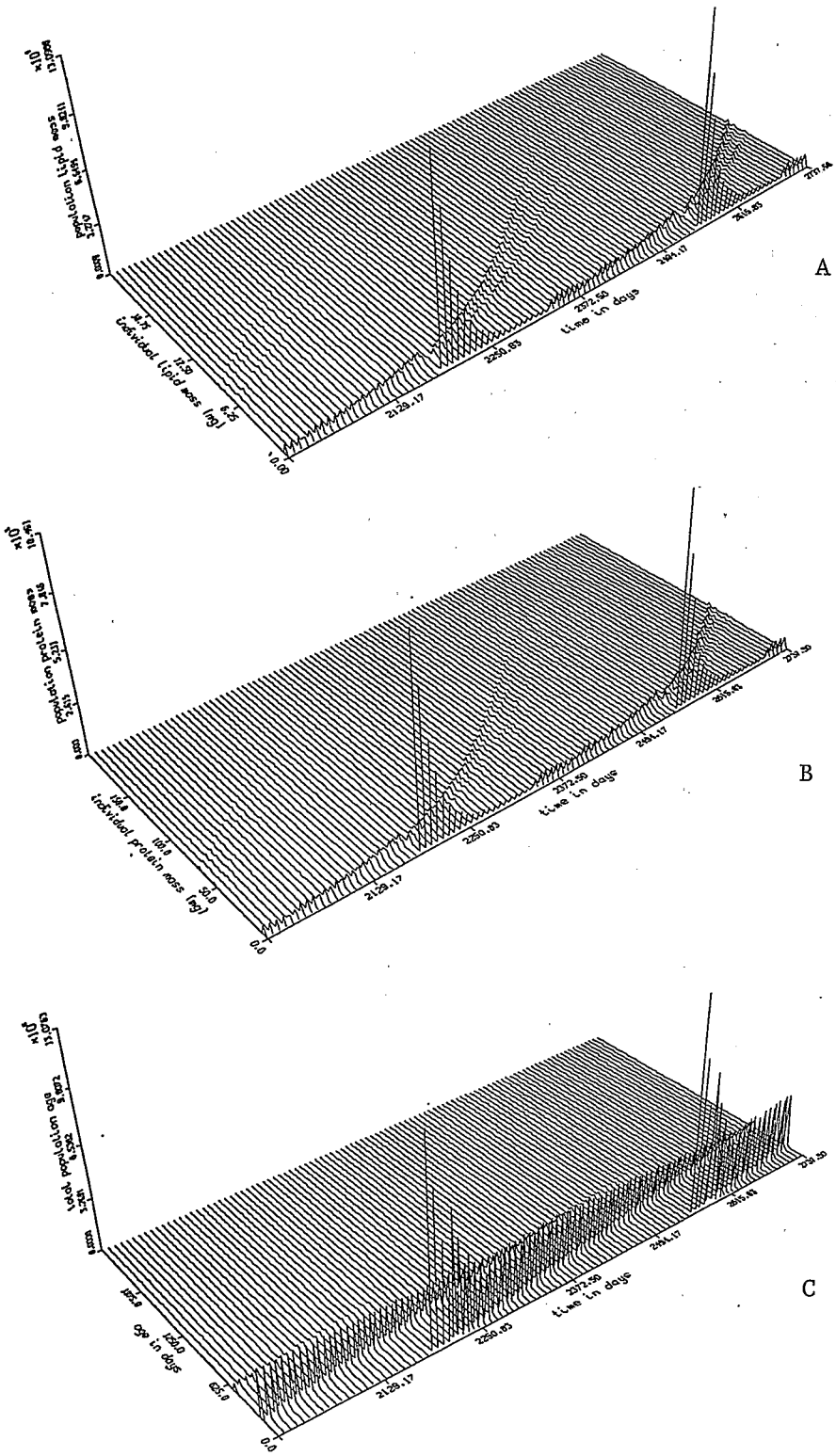


Figure 5. The graphs of nine ecotypes of fish utilized in structuring the population. [The differences between the ecotypes are the resource level at which they feed and the fraction of lipid in the food. Another variable used to generate variation in the population is the gut clearance rate; the variation caused by changes in this parameter are not presented here but they are used in the population model (smallest m_S).]


A
B
C
time in days
2128.17
2250.83
2372.50
2494.17
2615.83
2737.50
0.0
6.25
12.50
18.75
25.00
50.0
75.0
100.0
125.0
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175.0
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TOXIC EFFECTS AT THE POPULATION LEVEL

As we have noted, survival of the fattest is a theory obtained by considering toxic exposures to a static population-exposures that are necessarily acute. We have demonstrated in previous work that this theory is not valid when the hypothesis of a static population is violated.

Survival of a population after chronic exposure is determined not only by the lipid distribution as in the survival of the fattest but now, in a dynamic setting, also by the growth rate of the individuals in the population. When the model population was stressed by a chronic exposure at a concentration where there was some mortality but also some cohort survival, the particular ecotypes of the surviving individuals depended upon many factors including biological aspects of the population and toxicological aspects of the chemical. The biological features were related to the intrinsic oscillations of the population, to the assessment of mortality, and to the reproductive characteristics. Toxicological features of importance include the length of exposure, the initial time of exposure, as well as the strength of the chemical.

The fact that exposure may come from both the environmental and the food chain pathways complicates determination of cause of death or, equivalently, reason for survivorship in a dynamic population. We shall focus upon a single aspect of the exposure, namely its duration. This approach will illustrate our procedure to assess the effects of a toxic chemical on our model fish population. In each instance below we shall stress the population employing the same chemical but we investigate the outcomes of different durations of exposure.

The dynamics of the physiological variables--lipid, structure, and age--as expressed through the respective distributions of the unstressed population on the time interval from 2007.5 days to 2737.5 days are presented in Figure 6a, b, c. This and all subsequent population graphs are scaled by a maximal physiological variable size determined by the ecotypes. The purpose of this scaling is to allow viewing of the larger individuals in the population. Even with this scaling, the smaller individuals still dominate the graphics because of their numbers.

A 4-8 day exposure, initiated on day 2025, to a 3.2 parts-per-million concentration of a chemical having an octanol-water partition coefficient of 10^5 decreases the number of cohorts of fish in the stressed model population from 85 down to 32. The surviving cohorts primarily consist of older individuals because no individuals in the younger age classes survive the exposure. The population continues to persist until day 2502, at which time it goes to extinction. The reason for this extinction is that the numbers of individuals are small and the types of individuals grow sufficiently slowly so that a threshold biomass in the density dependent mortality function is not exceeded. Figure 7a, b, c represent the lipid, structural, and age distributions in this stressed population.

An exposure with the same chemical at the same concentration but of duration 3.8 days instead of 4-8 days leads to a completely different con-

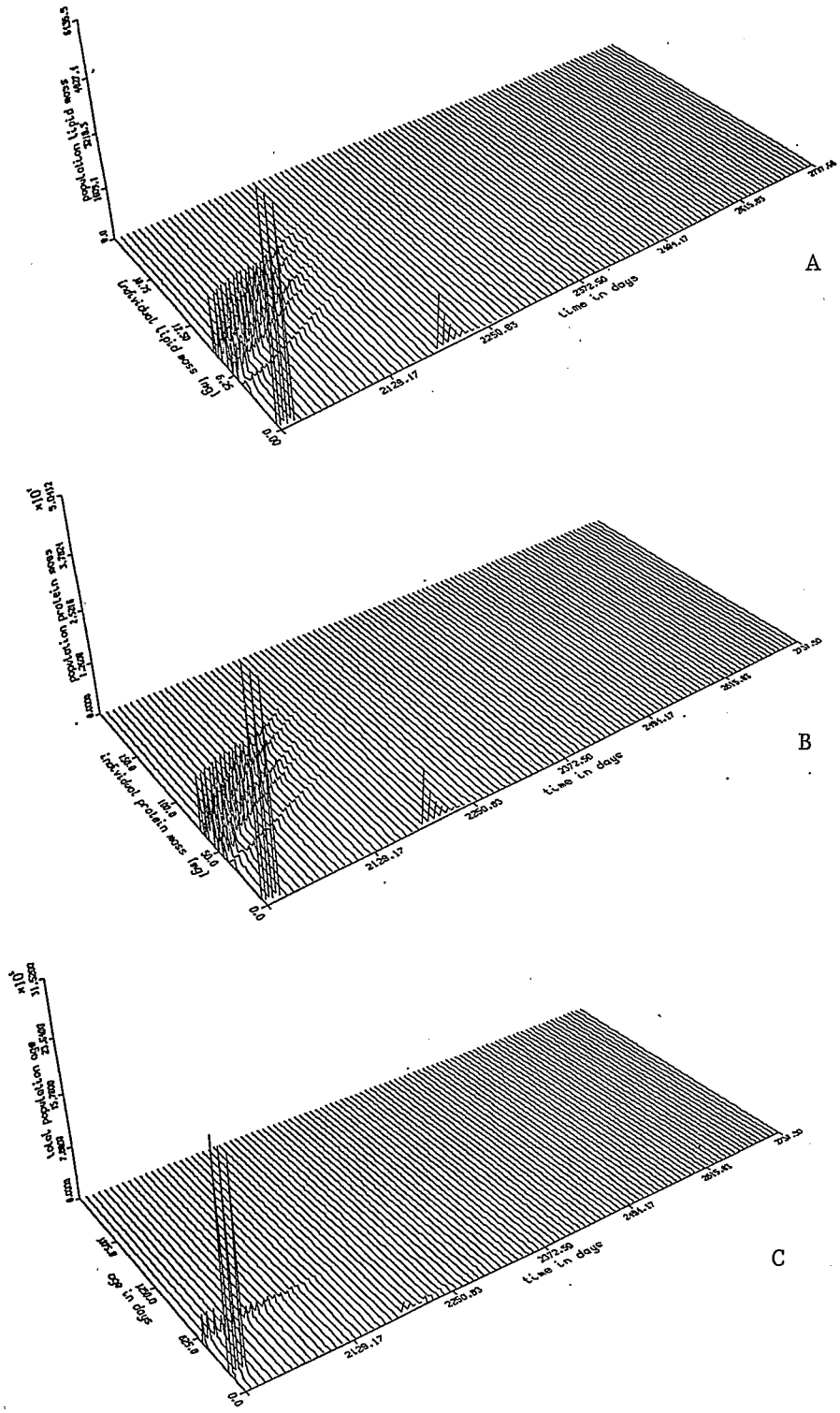

Figure 7. Extinction caused by chemical stress. [Duration of exposure is 4.8 days initiated on day 2025. See text for details about chemical properties. A. The dynamic lipid density of the stressed model population. B. The dynamic strusture density of the stressed model population. C. The dynamic age density of the stressed model population.]

clusion. This population is now persistent over the study period. The number of remaining cohorts that survive are fish that grow relatively fast. It is their presence that allows this population to survive and their absence in the above stressed population that contributes to its demise. Figure 8 demonstrates the evolution of this stressed population. A comparison with the unstressed population of Figure 6 shows relatively small differences in the stressed and the unstressed populations.

SUMMARY

To demonstrate a proposed protocol for assessing the effects of a toxicant on a fish population, we have developed a model of an individual fish and incorporated these individual dynamics into a population model. Accordingly, our approach focuses upon the individual--the interface between the biology and the chemistry. This allows investigation of the effects of toxicants on populations by accumulating the effects on individuals and reinforces our premise that the basis for studies in ecotoxicology should be the individual.

The model fish population was stressed by a chemical and the effects of the duration of exposure studied. The ecotypes of the surviving individuals were determined by both biological succession in the population and toxicological aspects of the chemical. Biological succession proceeds to the dominant--fastest growing--ecotype among all ecotypes that structure the population. Toxic stress can alter the successional dominant ecotype from the fastest grower ecotype (Hallam et al. 1989). Here we have seen that the duration of exposure can also drastically influence the population evolution. A situation was presented where the faster growing morphs were eliminated from the population and the population eventually went to extinction (Figure 7). A shortening of the duration of exposure allowed the population to persist (Figure 8). Even though the surviving cohorts were not the fastest growing morphs they grew sufficiently fast to overcome the threshold of mortality present in the density dependent mortality representation.

As we have previously demonstrated for *Daphnia* in a dynamic population model, survival of the fattest is not valid, even when exposure is only from the environmental pathway. For fish this statement is also valid. Both the cases represented by Figures 7 and 8 indicate a setting where the surviving individuals were not the fattest; they were the slower or the faster growers of the ecotypes. The "survival of the fastest" grower is characteristic of the evolutionary behavior of our model. The surviving ecotypes with the largest growth rate will eventually dominate the population.

The experiments necessary to study our hypotheses and our conclusions have not been completed and, in some instances, not even conceptualized. However, the models studied here are well grounded in biological and toxicological information and appear credible. It is a clear implication of this and related studies that risk assessment should not be based solely upon attributes of the toxic chemical. The biology of the exposed organisms is fundamental to the determination of the effects of the toxicant on a population.

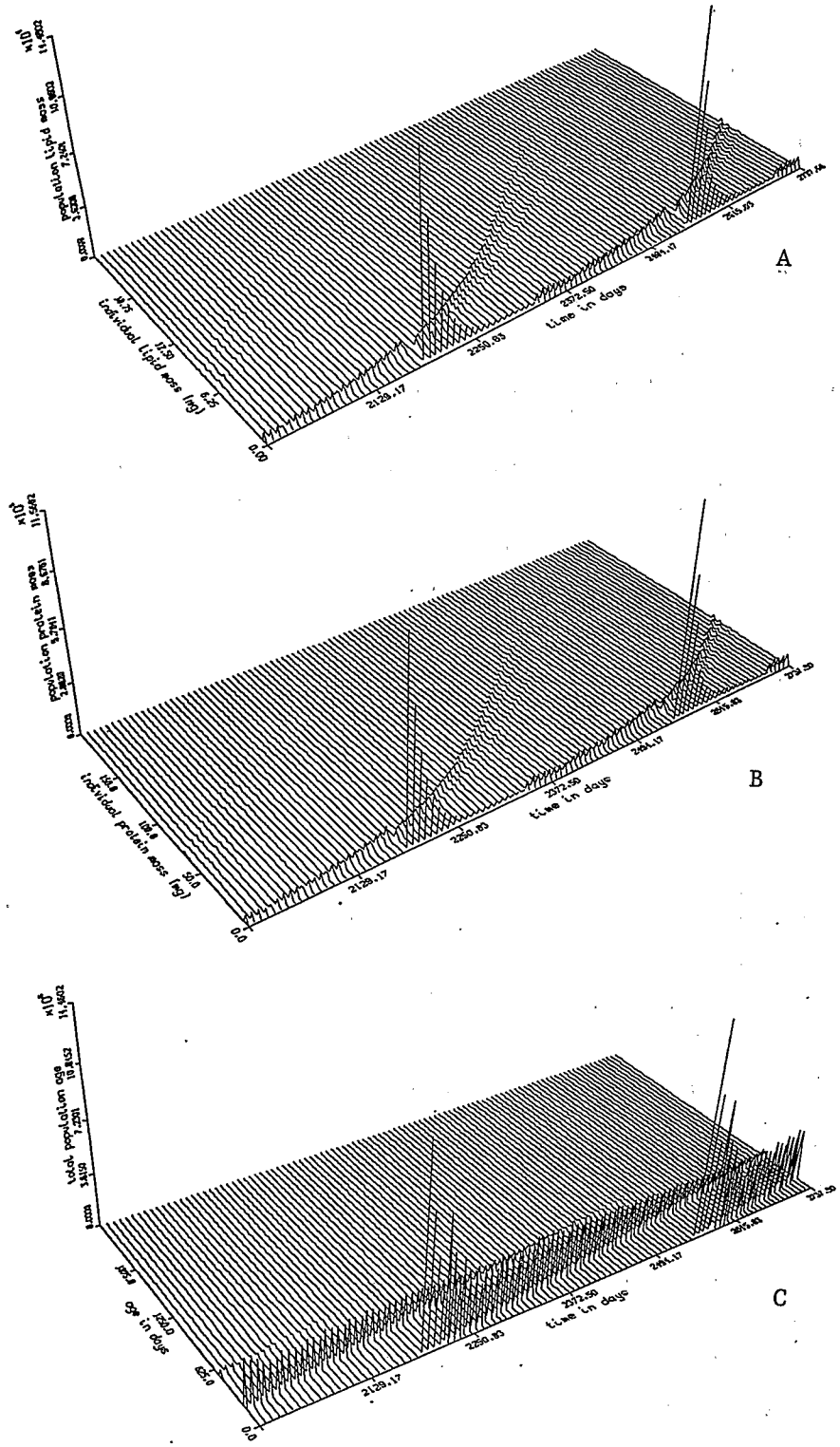

Figure 8. Population persistence following a chemical stress of 3.8 days duration initiated on day 2025. [The same chemical is used as in the situation represented by Figure 7. A. The dynamic lipid density function of the stressed model population. B. The stressed density function of the stressed model population. C. The dynamic age density function of the stressed model population.]

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TOXICITY OF ISOPROTHIOLANE TO FISHES

by

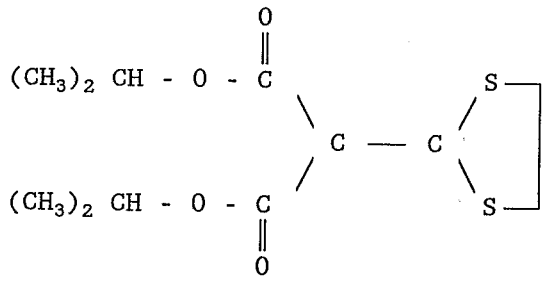
Zhai Liangan[1], Zhao Xiaochun, Yao Aiqin, and Li Jifang

INTRODUCTION

It is an inevitable trend that new pesticides will be substituted for organic chlorine ones because of the toxicity of the latter's residuals to environments, animals, fishes and even human beings, although they have played an important role in the control of pests and insects in China. In recent years, we have produced and imported organophosphors, pyrethrin, and other pesticides but reports about their toxicity to fish are quite scarce. This paper reports a toxicity experiment using a bacteriocide--Isoprothiolane. The goal is to provide a scientific base for pesticides application, environmental protection, and criteria and standards development for fisheries water quality.

MATERIALS AND METHODS

Isoprothiolane (IPT), i.e. Fuji--one, NNF--109, is a heterocyclic pesticide that was experimentally produced by Japan Pesticides Company in 1968. Since 1983, we have imported 2500 tons (95% emulsifiable concentrate). The name of its effective chemical composition is, bis(isopropyl)-1.3-dithiolane-2-ylidene-malonate, with a structure of



Molecular formula: $C_{12}H_{18}O_4S_2$, molecular weight: 290.4.

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[1]The Yangtze Institute of Fisheries, Chinese Academy of Fishery Sciences, PRC.

Purified isoprothiolane is a white crystal, and its melting point is 54°C. Its steam pressure is $1.4^1 \times 10^{-4}$mmHg, and it is slightly dissolved in water and easily in benzene, acetone, alcohol and other organic solvents. Its solubility in water at 20°C is 48 ppm. Isoprothiolane is a systemic bactericide for the control of rice blast, and can prevent physiological disorders caused by low temperature, over-moisture in soil, etc.

TABLE 1. TEST ORGANISMS

Species	Standards	Simple description	Source
Common carp	Average body length, 1.72 cm; weight, 0.0368 g	Omnivore, living in the lower layer	From the experimental fish farm of our institute
Silver carp	Average body length, 1.80 cm; weight, 0.046 g	Feeder on phytoplankton, living in the upper layer	From the experimental fish farm of our institute
Fish eggs of grass carp	---	From fertilized eggs to gastrula stage	From the experimental fish farm of our institute
Daphnia magna	2 to 3 days age	A kind of fish food feeding on phytoplankton	Cultured in laboratory

The water used in this experiment is tap water that has been exposed to air. Its turbidness is 3.7; pH 7.57; total hardness is 8.6 (Germany Hardness); dissolved oxygen meets fisheries requirements.

ACUTE TOXICITY EXPERIMENT WITH FISH

Concentrations of isoprothiolane for experiments with common carp (*Cyprius carpio*) were determined to be 8.0, 2.7, 0.9, 0.3, and 0.1 ppm by the equidistant transformation method of logarithm; those for silver carp (*Hypothalmichthys molitrix*) were 4.5, 2.5, 1.4, 0.8, and 0.45 ppm. Each group had a replicate and a control. Poisoning symptoms and the mortality rate of fishes were noted, then the 96-hour LC_{50} was obtained by the Kärber method. The results were multiplied by 0.1 to find the safety concentration.

GROWTH EXPERIMENT WITH FISH

The experiment carried out by stocking 10 fish in 0.4 ppm (safety concentration for common carp), 0.85 ppm, 0.45 ppm, 0.25 ppm (survival concentration for silver carp) and in pesticide free water (control). Fish were fed in fixed quantity for 28 days. The growth rate was determined by measuring the body length and weight at the end of the experiment in comparison with that at the beginning.

EMBRYONIC DEVELOPMENT EXPERIMENT

Concentrations of isoprothiolane for experimental groups were determined to be 8.00, 4.50, 2.50, 1.40 and 6.88 ppm by equidistant transformation method of logarithm. Twenty eggs at the gastrula stage were stocked in experimental groups and a control group. Embryo lethality, hatching rate and survival rate were noted periodically and compared with that of the control group.

EXPERIMENT WITH DAPHNIA MAGNA

Seven isoprothiolane concentrations were determined between 0.15 and 2.5 ppm, and a control and a replicate were set up. Ten *Daphnia magna* was stocked in each group. Mortality was noted at 24, 48 and 96 hours, and the LC_{50} was solved from those counts.

RESULTS AND DISCUSSION

In the acute toxicity experiment, the fish appear to be uncomfortable and to swim at the water surface and to be sluggish in behaviour at the beginning of the experiment. At high concentrations of isoprothiolane, fish would be blackened in body color and some even died. Results in Table 2 show that isoprothiolane has moderate toxicity to fish.

In the growth experiment (Table 3), isoprothiolane at 0.40 ppm had no effect on the growth of common carp, but 0.80 ppm had a distinct effect on the growth of silver carp. 0.45 ppm, 0.25 ppm and control had no effect on the growth of silver carp, so the maximum functioning concentration of isoprothiolane on silver carp growth is 0.45 ppm.

In the embryonic development test (Table 4), it was shown that 2.50 ppm, 4.50 ppm and 8.00 ppm could affect development. The embryonic body in 1.40 ppm, 0.80 ppm and control group begins to wriggle slightly after 12 hours. Hatching rates from high to low concentrations are 20%, 30%, 85%, 90%, 80% and 75%, respectively. Obviously, only 4.50 ppm and 8.00 ppm have a distinct effect on embryonic development. Fingerlings in those concentrations with high deformative rate all died after 96 hours. There is no significant difference among 2.50 ppm, 1.40 ppm, 0.80 ppm and control group. The maximum no-effect concentration for the embryo was 2.50 ppm.

In the daphnia experiment (Table 5), there was no significant difference among 0.15 ppm, 0.27 ppm, 0.47 ppm, 0.84 ppm, 1.50 ppm and control group. *Daphnia magna* should not be higher than 0.178 ppm.

TABLE 2. ACUTE TOXICITY EXPERIMENT RESULT OF IPT TO FISH

Species	Concen. ppm	Logarithmic value of Concen. (X)	No. of fish	Mortality (r)	r^2	96-hr LC_{50}
Common carp	8.0	0.903	10	10	100	4.138
	2.7	0.431	10	1	1	
	0.9	-0.046	10	0	0	95% confidence
	0.3	-0.523	10	0	0	limits
	0.1	-1.000	10	0	0	=4.138±0.888
	Total	---	--	$\Sigma r=11$	$\Sigma r^2=101$	SE=0.0477
Silver carp	4.5					3.180
	2.5					
	1.4					95% confidence
	0.8					limits
	0.45					=3.180±0.360
	0.25					SE=0.025
	Total					

TABLE 3. GROWTH EXPERIMENT RESULT OF IPT TO COMMON CARP AND SILVER CARP

Species	Concen. ppm	Beginning Length, cm	Beginning Weight, g	Ending Length, cm	Ending Weight, g	Increase Length, cm	Increase Weight, g	Explanation
Common Carp	0.40	1.72	0.0368	2.03	0.085	0.31	0.0482	
	0.00	1.72	0.0368	2.02	0.072	0.30	0.0352	27 days
Silver Carp	0.80	1.80	0.046	2.35	0.0742	0.55	0.0282	
	0.45	1.80	0.046	2.46	0.0940	0.66	0.048	
	0.25	1.80	0.046	2.44	0.0990	0.64	0.053	
	0.00	1.80	0.046	2.54	0.1175	0.74	0.072	28 days

TABLE 4. EMBRYONIC DEVELOPMENT RESULT OF IPT TO GRASS CARP

Concen.,	Number of Eggs	Survival number[a]							
		6hr	9hr	12hr	24hr	28hr	34hr	72hr	96hr
8.00	20	20	20	18	18	4	4	4	All died
4.50	20	19	19	18	17	6	5	5	5
2.50	20	19	19	19	19	17	17	15	14
1.40	20	20	20	20	18	18	18	17	17
0.80	20	20	20	20	17	17	17	17	16
Control	20	19	19	18	16	15	15	15	15

[a]At 110 hours, all had died.

SUMMARY

1. 96-hour LC_{50} values of isoprothiolane for common carp and silver carp are 4.138 ppm and 3.180 ppm, respectively. 96-hr LC_{50} value multiplied by 0.1 are 0.4138 ppm and 0.1380 ppm, respectively.

2. 0.45 ppm of iosoprothiolane is the maximum no-effect concentration for silver carp growth.

3. 4.50 ppm of isoprothiolane is the maximum no-effect concentration for embryonic development.

4. 96-hour LC_{50} value of isoprothiolane for Daphnia magna is 1.780 ppm. In conclusion, 0.318 ppm of isoprothiolane is a safe level with respect to acute toxicity, growth embryonic development of Daphnia magna survival. Therefore, it is advisable to recommend that 0.318 ppm (0.3 ppm) of isoprothiolane should be the water quality standards for fisheries.

TABLE 5. RESULT OF EXPERIMENT WITH *Daphnia magna* (96-hr)[a]

Concen., ppm	*Daphnia magna* number	Result				
		24 hr	48 hr	72 hr	96 hr	96 hr LC_{50}
2.50	10	10	10		0	
2.00	10	10	10		3	
1.50	10	10	10	9	8	
0.84	10	10	9	9	9	1.7826168
0.47	10	9.5	9	8.5	7.5	≈ 1.80
0.27	10	10	10	8	9	
0.15	10	10	9	8	9	
Control	10	10	10	9.5	9.5	

[a]Water temperature 20±5°C. Results are for two replicate experiments.

FISH ENZYMATIC INDICATORS IN AQUATIC TOXICOLOGY

by

Xu Lihong[1], Zhang Yongyuan, and Wang Deming

INTRODUCTION

The response of an intact animal, such as death or a change of reproductive behavior, has been used as an index in classical toxicological tests. Such responses, however, occur only when a toxicant concentration is relatively high. The indices are comparatively simple and approximate. More and more physiological and biochemical indices are being introduced into toxicology now.

By measuring changes in the activities of different enzymes, changes of metabolic or hematological parameters, or changes in the components of tissue of animals inhabiting a certain water body or being exposed to toxicants, a series of biochemical parameters can be obtained. Similarly, when selected organ or tissue homogenate is exposed to toxicants, *in vitro* biochemical parameters can be obtained. With these biochemical indices, toxicant action mode could be explained, health of animal in the water could be evaluated, early signs of potential pollution could be provided, fast screening of toxicity of chemicals could be made, and effects of long-term exposure to low toxicant dosage on an animal could be detected. Also, the biochemical indices are helpful for determining the target organ of toxicant action. Some indices can be used as specific indicators for the existence of certain pollutants.

Enzymes are one of the most important macromolecular proteins in the body. Some enzymes have already been used as toxicological indices, and others are potentially useful. The reaction between key enzymes of organisms and toxicants has become a topic of mutual interest to toxicologists and biochemists.

Adenosine triphosphatase (ATPase) plays an important role in ion transportation and osmoregulation of fish. Acetylcholinesterase (AChE) inactivated acetylcholine is released from the synamses of cholinergic nerves preventing

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tetanic firing of the post-synaptic nerve. The effects of toxicants on ATPase and AChE are presented in this paper. The possibility of the two enzymes being used as toxicological indices and their mode of action are discussed.

MATERIALS AND METHODS

Grass carp (Ctenopharyngodon idellus), 11 to 14.5 cm long, were caught in a fish pond and acclimated in dechlorinated tap water for 3 days. Paradise fish (Macropodus opercularis), 1-year old and 5.9 to 7.8 cm long, were cultured in a laboratory, where temperature was kept over 15°C. For ATPase assay (Paxton and Limminger 1983), the relevant tissue was homogenized in cold buffer (containing 40 mM imidazole, 250 mM sucrose, and 5 mM EDTA at pH 7.1 and 25°C). The homogenate was centrifuged at 3000 rpm for 5 minutes and the supernatant was kept for enzyme assay. Supernatant (0.2 ml) and 0.3 ml of tested toxicant stock solution (0.3 ml of double-distilled water substituted for the stock solution in the control and in in vivo tests) were incubated in a reaction mixture at 25°C for 20 minutes, then 0.1 ml Na_2 ATP was added to the mixture. The reaction mixture in a final volume of 3.0 ml contained 40 mM imidazole, 100 mM Na^+, 20 mM K^+, 2 mM Mg^+ (without Na^+ and K^+ for NaK-ATPase assay) and 2 mM Na_aATP (pH 7.1, 25°C). After 15 min, the reaction was stopped by 30% cold TCA, and the reaction mixture was centrifuged (3000 rpm) for 5 minutes. The supernatant was kept for inorganic phosphate determination. Protein concentration of the enzyme was estimated with the Lowry method (Scope 1982). The specific activity of the enzyme was recorded in terms of micromolar $Pimg^{-1}$ protein hr^{-1}. For AChE assay, brain tissue was homogenated in 0.01M phosphate buffer (pH 7.2). In the final homogenation, 2 mg of brain tissue per milliliter of homogenate was obtained. One milliliter of this homogenate was added to 1 ml of 0.004M bromine acetylcholine, then the mixture was incubated for 20 minutes at 30°C. The remaining bromine acetylcholine was measured according to the Hestrin method (Hestrin 1949). The specific activity of the enzyme was recorded in terms of micromolar bromine acetylcholine mg^{-1} brain tissue hr^{-1}.

In the Hg^{2+} treatment of grass carp, the fish were exposed to 0.2 ppm Hg^{2+} (15°C, pH 7.8). After 24 hours, NaKMg-ATPase activity of various tissues were measured for both the experimental group and the control group.

In the Cu^{2+} treatment of paradise fish, the fish was exposed to 0.1 ppm Cu^{2+} in a flow-through system (21-24°C, pH 7.2-8.0). After 4 weeks, brain AChE activity was measured. Control (2.8 $\mu gCu^{2+}/l$) was measured at the same time.

LAS (linear alkyl benzylsulfonate) and TCB (tri-chlorinated biphenyls) were used as environmental analysis standard reagents. Others were analysis reagents.

RESULTS

NaKMg-ATPase activities of grass carp caught at different times and accli-ated 3 days in the laboratory were measured. Table 1 shows NaKMg-ATPase activities of gill; Table 2 shows that of kidney. One-way variance analysis

TABLE 1. GILL NaKMg-ATPase ACTIVITIES OF GRASS CARP CAUGHT AT DIFFERENT TIMES

Body length, cm	Acclimated temperature, °C	ATPase activity, mean ± SD
11.5-12	19.3-20.5	18.69 ± 1.09
12.5-13.5	13.0-13.5	19.69 ± 1.00
12-12.5	13	17.41 ± 3.15
11.5-13.5	12.2-14.0	15.44 ± 1.12
12.5-13.5	14.5	15.57 ± 1.07
13-13.5	17.2-19.0	16.25 ± 2.33
11-12	19.2-20.2	18.28 ± 1.95
13.5-14	15.5-18	19.58 ± 3.48

was carried out. When the significance level was 0.05, there is no significant difference in activities of NaKMg-ATPase for both gill and kidney between different groups of fish.

Plotting the probit of percentage of inhibition of toxicants on enzyme and logarithmic concentration of toxicants, we obtained straight lines (Figure 1). I_{50}, 50% inhibition concentration of toxicant on ATPase, was calculated from a linear equation.

a: Hg^{2+} $I=4.70+0.51 \ln C$ I_{50} 1.8 ppm (kidney)
b: LAS $I=4.14+0.63 \ln C$ I_{50} 3.9 ppm (gill)
c: TCB $I=3.39+0.55 \ln C$ I_{50} 18.4 ppm (gill)
d: Cd^{2+} $I=1.56+0.71 \ln C$ I_{50} 127.2 ppm (kidney)

I is the probit of percentage of inhibition of toxicant. C is the concentration of toxicant.

Effects of Hg^{2+} on NaKMg-ATPase, NaK-ATPase, and Mg-ATPase are shown by plotting inhibition percentage and toxicant concentration in Figure 2. As evident from the figure, the inhibition of Hg^{2+} on NaK-ATPase was the strongest.

After 24 hours of exposure to Hg^{2+}, gill and kidney NaKMg-ATPase in grass carp were measured and the results are shown in Table 3. At the significance level of 0.05, there is no significant difference between the test and the control groups.

TABLE 2. KIDNEY NaKMg-ATPase ACTIVITIES OF GRASS CARP CAUGHT AT DIFFERENT TIMES

Body length, cm	Acclimated temperature, °C	ATPase activity, mean ± SD
11.5-12	19.3-20.5	20.25 ± 0.94
12-125	12-13	26.09 ± 0.75
11.5-13.5	12.2-14	24.29 ± 4.12
12.3-13	17.2-19	24.61 ± 1.46

After 4 weeks of exposure to Cu^{2+}, brain AChE activities of paradise fish were measured and the results are presented in Table 4. At the significance level of 0.05, there is no significant difference between the test and the control groups. It seems that the non-neurotoxic chemical has no effect on AChE.

DISCUSSION

Since the 1960s, studies of the reaction of ATPase to toxicants both *in vivo* and *in vitro* have been carried out in large numbers by scientists. Different organisms were used as experimental materials, a wide variety of chemicals were studied, and some work even delved into the study of enzyme dynamics. In these studies, the probable mechanisms of the effect of toxicants on ATPase have been scrutinized, and the mode of intoxication and the target organ of the toxicants were deduced. Because quite a number of chemicals can influence ATPase, such as organochlorine pesticide, heavy metals, surfactants, DEHP, PCBs and petroleum refinery wastewater (Boese et al. 1982, Davis et al. 1972, Kuhnert and Kuhnert 1976, Riadel and Christensen 1979, Srivastava et al. 1975, Verma et al. 1979a, Verma et al. 1979b, Yap et al. 1971), some investigators proposed that ATPase activity could be used as a nonspecific indicator in aquatic environment monitoring (Haya and Waiwood 1983). Because of the sensitivity of ATPase to toxicants, many investigators thought that ATPase activity could reflect the damage of a sublethal concentration toxicant to an organism and predict potential pollution of chemicals in the aquatic environment.

There is a major premise that fish ATPase could be an indicator. ATPase plays a key role in ionic and osmotic regulation in fish body, so changes in ATPase activity might reflect the health or even the survival of the fish.

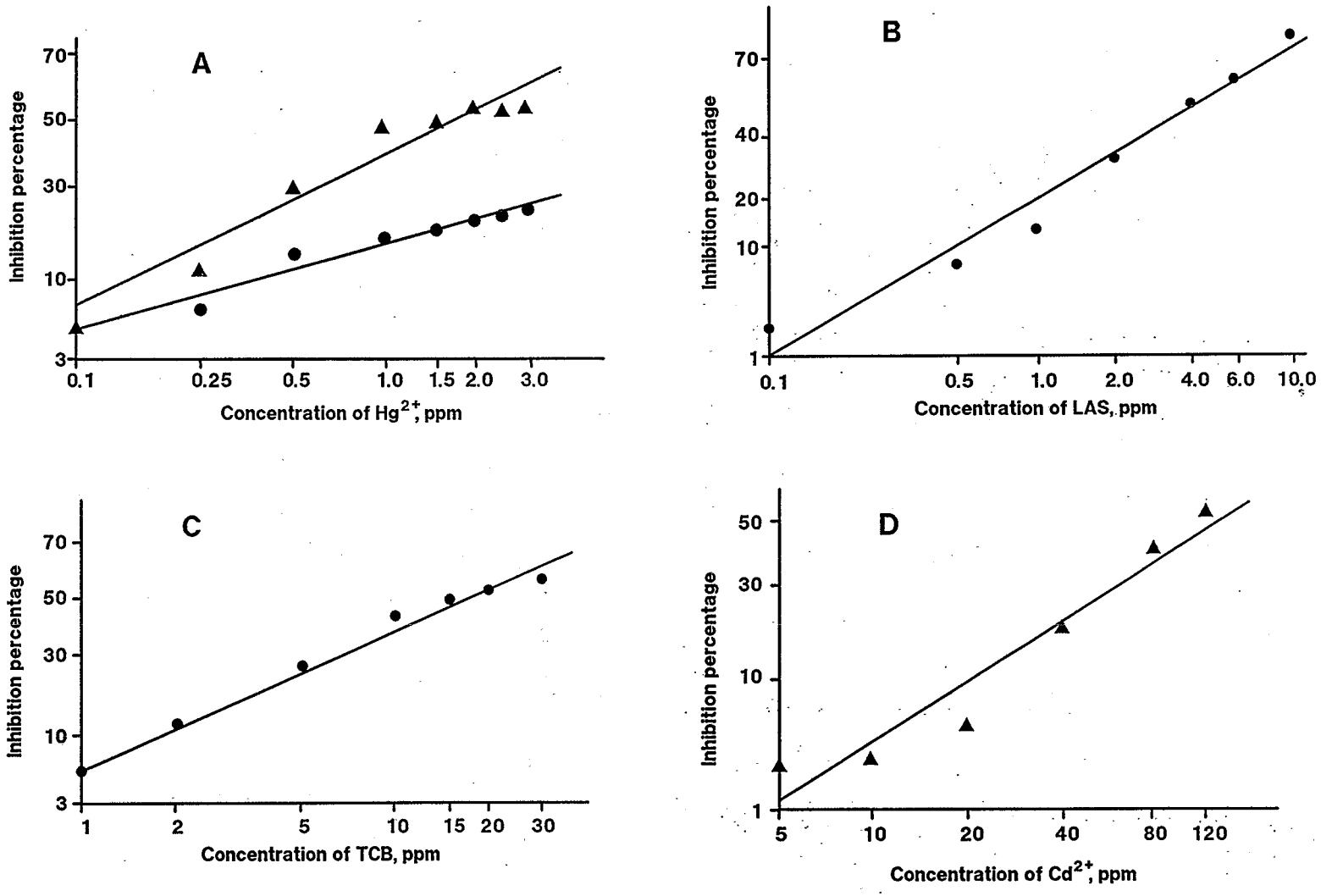

Figure 1. Inhibition of toxicants on grass carp gill and kidney NaKMg-ATPase.

TABLE 3. IN VIVO EFFECT OF Hg^{2+} ON GRASS CARP GILL AND KIDNEY ATPase (n=4)

	Specific activity, mean ± SD	
	gill	kidney
control	17.05 ± 3.15	22.38 ± 5.28
0.2 ppm Hg^{2+}	16.48 ± 4.54	24.08 ± 5.83

After 3 days acclimation in the laboratory, there is no significant change in ATPase activity of fish caught from fish-pond. This is important for the in vivo tests. When an in vitro test is carried out, only a small quantity of tissue is needed. So, from the view of experimental material, it is practicable to use grass carp tissue as an enzyme source or to carry out in vivo tests with grass carp.

All the toxicants studied in our tests have obvious effects on ATPase under in vitro conditions. Such regularities were found on the basis of the effects of Hg^{2+}, LAS, TCB, CD^{2+} on ATPase, that is, linear relationships were obtained from the plotting of probit of inhibition percentage and logarithm of toxicants concentration. This is the same as found in biological tests. This means that an in vitro test does show some characteristics of the toxic action.

Hg^{2+} had greater inhibition on kidney NaKMg-ATPase than on gill NaKMg-ATPase. In Figure 2, it is demonstrated that Hg^{2+} had stronger inhibition on NaK-ATPase than on Mg-ATPase. NaK-ATPase activity in the kidney is higher than in the gill in freshwater fish (Jampol and Epstein 1970).. So, when NaKMg-ATPase was used as an index, the change of kidney NaKMg-ATPase is more sensitive than that of gill NaKMg-ATPase. This indicates the need to search for several tissues in order to select the most sensitive indicator. For the same reason, Cd^{2+} had great inhibition on kidney NaKMg-ATPase than on gill NaKMg-ATPase. Considering the properties of NaK-ATPase on the one hand and metal on the other hand, metal should affect NaK-ATPase more readily than Mg-ATPase.

After 24 hours of exposure to 0.2 ppm Hg^{2+}, grass carp gill and kidney NaKMg-ATPase were unaffected and death occurred at 0.4 ppm Hg^{2+} exposure. Sastry and Sharma (1980) reported the results of this experiment in which freshwater fish were exposed to 1.8 ppm Hg_+ for 96 hours and to 0.3 ppm Hg^{2+} for 15 days and 30 days. ATPase activity increased a little in the 96-hour acute exposure but decreased slightly in the 15-day and 30-day exposures, although death occurred in all of the test groups. Maybe the lethal toxicity

TABLE 4. IN VIVO EFFECT OF Cu^{2+} ON PARADISE FISH BRAIN AChE

	Specific activity, mean ± SD (mean ± SD)
control	1.79 ± 0.36 (n=6)
100 $\mu gCu^{2+}/l$	1.47 ± 0.20 (n=5)

of Hg^{2+} to fish was not the inhibition on gill and kidney ATPase but its effect on other enzymes or tissues. For example, death probably resulted from the suffocation of fish owing to the deposition of Hg^{2+} on gill epithelia, a situation that would not be revealed in the in vitro test. So it is helpful to integrate the in vitro and the in vivo test results in deciding the probable mode of toxicity on organisms.

From previous reports and the present research, it is obvious that ATPase activity is affected by varied chemicals and a consistent pattern can be obtained. The 50% inhibition concentration of toxicant on enzyme (I_{50}), corresponding to LC_{50}, also could be determined. So, it is feasible to use ATPase activity as a quick nonspecific index, which is very helpful in toxicity evaluation of chemicals. Although I_{50} derived from an in vitro test cannot tell all the characteristics of chemicals, it does provide preliminary judgment for the toxic properties of chemicals. When ATPase activity of fish exposed to toxicants of low dosage is measured, sublethal effects can be detected, which provides another basis for understanding the toxic properties of chemicals.

AChE has been the earliest enzyme adopted in environmental monitoring (Weiss 1989). In the late 1940s, the action pattern of organophosphorus pesticide was found to provide specific inhibition on AChE. Since then, AchE has been used as an index showing the presence of organophosphorus pesticide. Compared with ATPase, AChE is a relatively specific indicator. It can only reflect the action of neurotoxic materials. A specific indicator like AChE is very important in toxicological research and environmental monitoring.

Certain biochemical indices represent particular functions. For example, the change of ATPase shows the status of the energy metabolism system in the body, AChE reflects the nerve impulse conduction of organism. Various physical function are reflected from specific indices. Life is maintained by various activities. Any process may have ill or even lethal effects on an organism. Therefore, one or several indices would not be enough to evaluate the whole effect of a chemical on an organism. Death and growth retardation, of course, can explain in some way the toxicity of a chemical, but no comprehensive evaluation can be made from them. Similarly, the change of ATPase

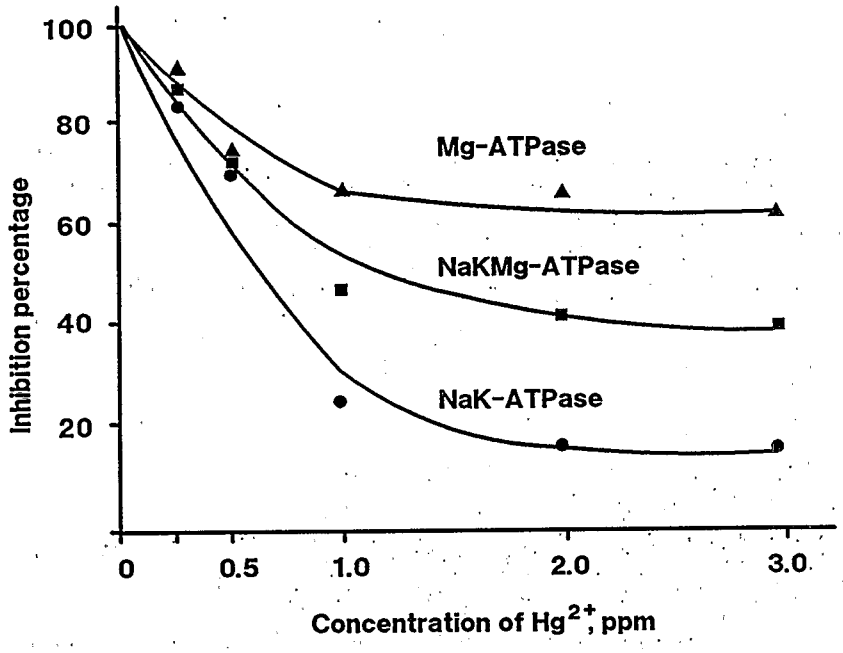

Figure 2. Inhibition of Hg^{2+} on grass carp kidney NaKMg-ATPase, NaK-ATPase, and Mg-ATPase.

and AChE cannot reflect the harmfulness of a chemical in a comprehensive way. Consequently, a series of indices that could represent different activities of an organism should be obtained for the comprehensive evaluation of toxicity of chemicals. After establishing the indices, chemicals could be classified according to the extent of their effects on different indices, e.g., chemicals having greater effects on ATPase could be classified as "ATPase-toxin" toxicants, those having greater effects on AChE, "AChE-toxic" toxicant, etc. Then, not only toxic properties will be understood, but also a certain knowledge of the action pattern can be gained. Comprehensive biochemical indices also can provide parameters for water quality criteria, as well as the basis and method for rapid screening of chemicals.

SUMMARY

Several kinds of toxicants had effects on grass carp ATPase. Plotting of probit of inhibition percentage and logarithm of toxicant concentration presents a linear relationship. I_{50} obtained from the plotting is a specific index of toxicant. Combined with other indices, I_{50} can be used in a screening test of chemicals. Cu^{2+} had no effect on paradise fish brain AChE. AChE is a relatively specific indicator, which can be used as an indicator monitoring pollution of neurotoxic toxicants.

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RIVER NETWORK WATER QUALITY MODELING USING THE ENHANCED STREAM WATER QUALITY MODEL QUAL2E

by

Qian Song[1]

INTRODUCTION TO QUAL2E

QUAL2E is a comprehensive and versatile stream water quality model. It can simulate up to 13 water quality constituents in any combination desired by the user. Constituents that can be simulated are:

1. Dissolved oxygen
2. Biochemical oxygen demand
3. Temperature
4. Algae as chlorophyll a
5. Organic nitrogen as N
6. Ammonia as N
7. Nitrite as N
8. Nitrate as N
9. Organic phosphorus as P
10. Dissolved phosphorus as P
11. Coliforms
12. Arbitrary nonconservative constituents
13. Three conservative constituents

The model is applicable to dendritic streams that are well mixed. It assumes that the major transport mechanisms, advection and dispersion, are significant only along the main direction of flow (longitudinal axis of the stream or canal). It allows for multiple waste discharges, withdrawals, tributary flows, and incremental inflow and outflow. It also has the capability to compute required dilution flows for augmentation to meet any prespecified dissolved oxygen level.

The computer program permits simulation of any branching, one-dimensional stream. The first step in modeling a system is to subdivide the stream system into reaches, which are stretches of a stream that have uniform hydraulic characteristics. Each reach is then divided into computational elements of equal length. Thus, all reaches must consist of an integer number of computational elements.

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There are seven different types of computational elements in the original model: (1) headwater element, (2) standard element, (3) element just upstream from a junction, (4) junction element, (5) last element in system, (6) input element, and (7) withdrawal element.

By using QUAL2E, the stream can be conceptualized as a string of completely mixed reactors (computational elements) that are linked sequentially to one another via the mechanisms of transport and dispersion.

The basic equation solved by QUAL2E is the one-dimensional advection-dispersion mass transport equation, which is numerically integrated over space and time for each water quality constituent. This equation includes the effects of advection, dispersion, dilution, constituent reactions and interactions, and sources and sinks. For any constituent, C, this equation can be written as:

$$\frac{\partial M}{\partial t} = \frac{\partial (A_x D_L \frac{\partial C}{\partial x})}{\partial x} dx - \frac{\partial (A_x \bar{u} C)}{\partial x} dx + (Axdx)\frac{dc}{dt} + S \qquad (1)$$

where

M = mass
x = distance (L)
t = time (T)
C = concentration (ML^{-3})
A_x = cross-sectional area (L^2)
D_L = dispersion coefficient (L^2T^{-1})

$\bar{u}$ = mean velocity (LT^{-1})

s = external sources or sinks (MT^{-1})

The constituent reactions and interreactions are linked by dissolved oxygen concentration. Figure 1 illustrates the conceptualization of these interactions. The arrows on the figure indicate the direction of normal system progression in a moderately polluted environment; the directions may be reversed in some circumstances for some constituents.

The mathematical relationships that describe the individual reactions and interactions are presented by Brown and Barnwell (1985).

The classical implicit backward difference method is used to solve Equation 1. The finite difference scheme is formulated by considering the constituent concentration, C, at four points in the mnemonic scheme, as shown in Figure 2. Three points are required at time n+1 to approximate the spatial derivatives. The temporal derivative is approximated at distance step i. After two steps differentiations, we obtain the equation:

$$a_i C_{i-1}^{n+1} + b_i C_i^{n+1} + c_i C_{i+1}^{n+1} = Z_i \qquad (2)$$

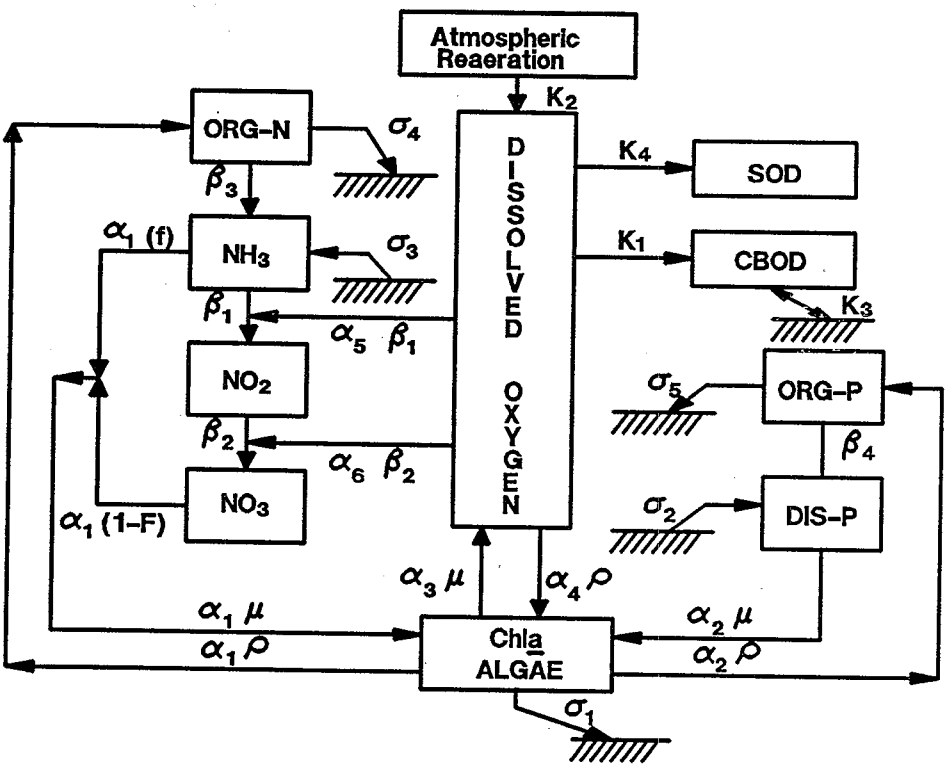

Figure 1. Major constituent interactions in QUAL2E.

where $a_i = -[(AD_L)_{i-1} \frac{\Delta t}{Vi\Delta x_i} + \frac{Q_{i-1}\Delta t}{V_i}]$

$$b_i = 1.0 + [(AD_L)_i + (AD_L)_{i-1}] \frac{\Delta t}{V_i \Delta x_i} + Q_i \frac{\Delta t}{\Delta v_i} - r_i \Delta t$$

$$c_i = -[(AD_L)_i \frac{\Delta t}{V_i \Delta x_i}]$$

$$Z_i = C_i^n \frac{S_i \Delta t}{V_i} + P_i \Delta t$$

The values of a_i, b_i, c_i, and z_i are all known at time n, and the $c^{n+1}{}_i$ terms are the unknowns at time step n+1. An efficient method for solving these simultaneous linear equations is presented by Brown and Barnwell (1985).

IMPROVEMENT OF QUAL2E

One of the limits in using QUAL2E is that the river system to be simulated can only have one last element. That means that the river system

cannot have branching elements or "fork" elements, from which simulated tributaries flow out from a main stem. And on the other hand, to improve the water quality, we need to know how much pollutant the river system could receive while the water quality of the river system still meets the required level, so that we can know at least how much pollutant should be removed from the waste discharge.

To model river system with fork elements, we first need to know the hydraulics of both the main stem and the tributary. We assume that the hydraulics of the river network are known. (For the fork element, the ratio of the flow of the main stem and the flow of the tributary, FLWRT are given.)

We know that, for a standard element i, the basic differential equation is:

$$a_i C_{i-1} + b_i C_i + c_i C_{i+1} = Z_i \tag{3}$$

in the case of a fork element, the basic equation becomes

$$a_i C_{i-1} + b_i C_i + c_i' C_{i+1} + f_j C_j = Z_i \tag{4}$$

where: $c_i' = c_i * FLWRT(I)$

$f_j = c_i * [1 - FLWRT(I)]$

Two methods can be used to solve these equations. Because c_i and f_j are several orders smaller then a_i and b_i, the first method is to assume that C_{i+1} equals C_j, and divide Equation 4 into two equations:

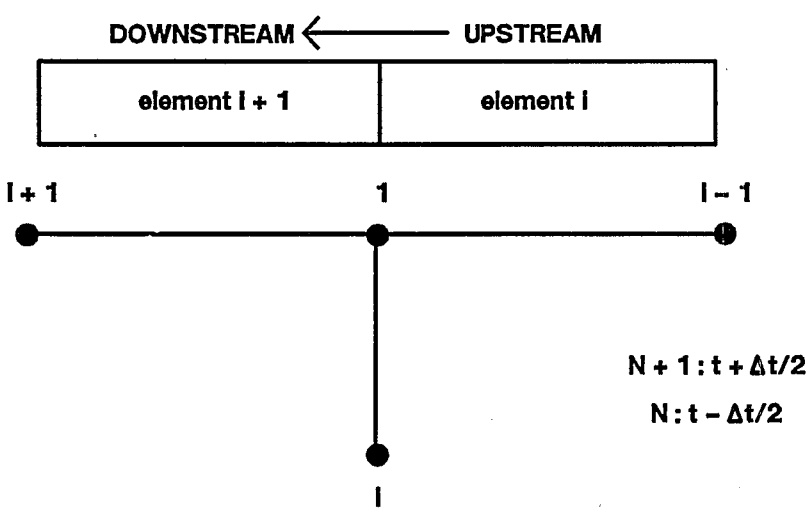

Figure 2. Classical implicit nodal scheme.

$$a_iC_{i-1}+b_iC_i+c_iC_{i+1}=Z_i \tag{5}$$

and:

$$a_iC_{i-1}+b_iC_i+c_iC_j=Z_i \tag{6}$$

Now it is easy to use the method presented by Brown and Barnwell (1985) to solve the simultaneous linear equations. When back-substituting the concentration from the last element, Equation 4 is used to solve the fork element concentration.

The second method does not use the foregoing assumption because, in many cases, C_{i+1} does not equal C_j.

Mathematically speaking, simultaneous linear equations are always solvable, but their efficient solution is not easy. In this paper, an efficient method for solving those equations is presented.

The method presented by Brown and Barnwell (1985) is applied before the fork element(s), and when a fork element is met in the process of forward substitution, we apply a sub-loop of forward and backward substitution, which is shown as follows

$$C_{i-1} + W_{i-1}C_i = G_{i-1} \tag{7}$$

$$a_iC_{i-1}+b_iC_i+c_i\ 'C_{i+1}+f_jC_j=Z_i \tag{8}$$

substituting Equation 7 into Equation 8, we obtain:

$$C_i + W_iC_{i+1} = G_i - F_iC_j \tag{9}$$

where: $W_i = c_i\ '/(b_i - a_iW_{i-1})$

$G_i = (Z_i - a_iG_{i-1})/(b_i - a_iW_{i-1})$

$F_i = f_j/(b_i - a_iW_{i-1})$

and substituting Equation 9 to the next basic equation and forward substituting the obtained equation, we have:

$$C_k + W_kC_{k+1} = G_k - F_kC_j \tag{10}$$

where: $F_i = f_j/(b_i-a_iW_{i-1})$, $F_k = a_kF_{k-1}$

$k = i+1, i+2, \ldots, n-1, n$

n = the order of the last element of the reach.

when $k = n$, $k+1 = kk$, and kk is the element order of the next type 3 or type 5 element, Equation 10 becomes:

$$C_n + W_n C_{kk} = G_n - F_n C_j$$

The forward substitution stopped when the next type 3 or type 5 element is met. Then back substitution is started from the element. The back substitute equations are shown as follows

$$C_k = A_k - B_k C_j - H_k C_{kk} \tag{11}$$

where: $A_k = G_k - A_{k+1} W_k$, $B_k = F_k - B_{k+1} W_k$, $H_k = -W_k H_{k+1}$

$A_n = G_n$, $B_n = F_n$, $H_n = W_n$

$k = n-1, n-2, \ldots, i+1, i$

when the back substitution stopped at the element i+1, we have:

$$C_{i+1} = A_{i+1} - B_{i+1} C_j - H_{i+1} C_{kk} \tag{12}$$

Substituting Equations 12 and 11 into the equations for the tributary, we have:

$$C_k + W'_k C_{k+1} = G'_k - F'_k C_{kk} \tag{13}$$

where: $W'_k = c_k/MM$, $G'_k = (Z_k - a_k G'_{k-1})/MM$, $F'_k = -a_k F'_{k-1}$,

$MM = B_k - a_k - a_k W'_{k-1}$, $W'_j = c_j/(b_j - a_j W'_i)$,

$G'_j = (Z_j - a_j G'_i)/(b_j - a_j W'_i)$, $F_j = -a_j F'_i$,

$k = j+1, j+2, \ldots, kk-2, kk-1$

When $k = kk-1$, we have:

$$C_{kk-1} + W'_{kk-1} C_{kk} = G'_{kk-1} - F'_{kk-1} C_{kk}$$

or $$C_{kk-1} = E_{kk-1} - L_{kk-1} C_{kk} \tag{14}$$

where: $E_{kk-1} = G'_{kk-1}$, $L_{kk-1} = F'_{kk-1} + W'_{kk-1}$

The back substitution of Equation 14 obtained the relationship of C_j and C_{kk}:

$$C_j = E_j - L_j C_{kk} \tag{15}$$

where: $E_k = G'_k - W'_k E_{k+1}$, $L_k = F'_k - W'_k L_{k+1}$

$k = kk-2, kk-3, \ldots, j-1, j$

Substituting Equation 15 to Equation 10, we have:

$$C_n + W'_n C_{kk} = G_n' \tag{16}$$

where: $W'_n = W_n - F_n L_j$, $G'_n = G_n - F_n E_j$

and rewriting Equation 14:

$$C_{kk-1} + L_{kk-1}C_{kk} = E_k$$

Now both the main stream and the tributary down stream from the fork element are ready to go back to the main loop of the forward and backward substitution.

By adding another two kinds of computational element--type 9 (fork) and type 8 (element just downstream from a fork on the tributary)--the QUAL2E source code is modified so that it could simulate the net-like river systems.

To calculate the maximum point source loads, we first need to linearize Equations 2. The process of linearization is shown as follows

(1). Assume that in one reach, the concentration of the constituent at the last element is equal to the concentration of the first element of the next reach:

$$C_n = C_{n+1} \tag{17}$$

(2). Let $A_{n-1} = Z_n/a_n$

$$B_{n-1} = -(b_n+c_n)/a_n$$

$$A_{n-2} = (Z_{n-1}-b_{n-1}A_{n-1})/a_{n-1}$$

$$B_{n-2} = -(b_{n-1}B_{n-1}+c_{n-1})/a_{n-1}$$

and

$$A_i = (Z_{i+1}-b_{i+1}A_{i+1}-c_{i+1}A_{i+2})/a_{i+1}$$

$$B_i = -(b_{i+1}B_{i+1}+c_{i+1}B_{i+2})/a_{i+1}$$

where: n = the number of element of the reach;

$i = 1, 2, 3, \ldots, n-3$

then we have:

$$C_1 = A_1 + B_1C_n \tag{18}$$

where: C_1 = the concentration of the constituent at the first element of the reach

C_n = the concentration of the constituent at the nth element of the reach.

Rewrite Equation 18 as:

$$C_n = A_i + B_iC_1 \tag{19}$$

where: $A_i = -A_1/B_1$ for reach i

$B_i = 1/B_1$ for reach i

After the linearization, we put the first basic equation of every reach together as a set of simultaneous equations. Substituting Equation 19 into these simultaneous equations, we have:

$$a_i C_{i-1} + b_i C_{i,1} + c_i C_{i,2} = Z_i \tag{20}$$

where: C_{i-1} = the concentration of the last element of reach i-1

$C_{i,1}$ = the concentration of the first element of reach i

$C_{i,2}$ = the concentration of the second element of reach i

Because c_i is several orders smaller, and the b_i and a_i, and $C_{i,1}$ and $C_{i,2}$ are not of very much difference, we assume that $C_{i,2} = C_{i,1}$. Substituting Equation 19 for Equation 20, we have:

$$a_i(A_{i-1} + B_{i-1}C_{i-1,1}) + (b_i + c_i)C_{i,1} \text{ and } Z_i \tag{21}$$

or $$AA_i C_{i-1,1} + BB_i C_{i,1} = ZZ_i$$

where: $AA_i = a_i B_{i-1}$, $i = 2,3,\ldots,n$

$BB_i = b_i + c_i$, $i = 1,2,\ldots,n$

$ZZ_i = Z_i - a_i A_{i-1}$, $i = 1,2,\ldots,n$

Rewriting Equation 21 in matrix form,

$$AB * C = Z_1 + Z \tag{22}$$

where:

$$AB = \begin{vmatrix} BB_1 & & & & & & & & & & & & & & & \\ AA_2 & BB_2 & & & & & & & & & & & & & & \\ 0 & AA_3 & BB_3 & & & & & & & & & & & & & \\ 0 & 0 & AA_4 & - & & & & & & & & & & & & \\ \vdots & \vdots & 0 & & - & & & & & & & & & & & \\ \vdots & \vdots & \vdots & & & - & & & & & & & & & & \\ \vdots & \vdots & \vdots & & & & - & & & & & & & & & \\ \vdots & \vdots & \vdots & & & & & - & & & & & & & & \\ 0 & 0 & 0 & - & - & - & - & - & - & - & - & - & - & - & - & BB_n \end{vmatrix}$$

$$C = (C_{1,1}, C_{2,1}, \ldots, C_{n,1})^T$$

$$Z_1 = (0, -a_2A_1, -a_3A_2, \ldots, -a_nA_{n-1})^T$$

$$Z = (Z_1, Z_2, \ldots, Z_n)^T$$

Under steady-state conditions,

$$Z_i = S_i/V_i + P_i$$

where: S_i = external sources or sinks

P_i = internal sources or sinks

Let $P = (p_1, p_2, \ldots, p_n)^T$, $Z_2 = Z_1 + P$, $S = (S_1, S_2, \ldots, S_n)^T$ and

$$V = \begin{vmatrix} v_1 & & & & & \\ & v_2 & & & & \\ & & v_3 & & & \\ & & & \vdots & & \\ & & & & \vdots & \\ & & & & & v_n \end{vmatrix}$$

Substituting Z, Z_1, P_1, S_1, and V into Equation 22 and rearranging the equation, we have:

$$S = V * AB * C - V * Z_2 \tag{23}$$

When substituting the river water quality criteria C^* to Equation 23, we have the maximum point load allowed in the river network:

$$S^* = V * AB * C^* - V * Z_2 \tag{24}$$

The first step in computing the maximum point source load is to redefine the reaches of the river system. The input element is the first element of the reach.

Using Equation 19 to Equation 24, an additional subroutine was coded for QUAL2E, which was called MAXCNT. Using the improved model, a case study is performed.

Figure 3 shows the stream network of computational elements and reaches. The river system contains one fork element. The input data set for the simulation is a modification from one of the demonstration input data sets shown at the US EPA's QUAL2E workshop at Nanjing University in 1985. Figure 4 shows the result of BOD simulation. Figure 5 shows the river system and the result of the BOD simulation of the above-mentioned workshop demonstration. Table 1 shows the BOD point source loads (P.L.) and the simulation result (concentration) at the first element of the reach. When substituting the concentrations in Table 1 as C^*, the maximum point source loads (MPL) of the river network should be the point source loads in Table 1. Table 2 shows the result.

REFERENCE

Brown, L. C. and T. O. Barnwell. 1985. Computer Program Documentation for the Enhanced Water Quality Model QUAL2E. U.S. Environmental Protection Agency, Athens, GA. EPA/600/3-85/065.

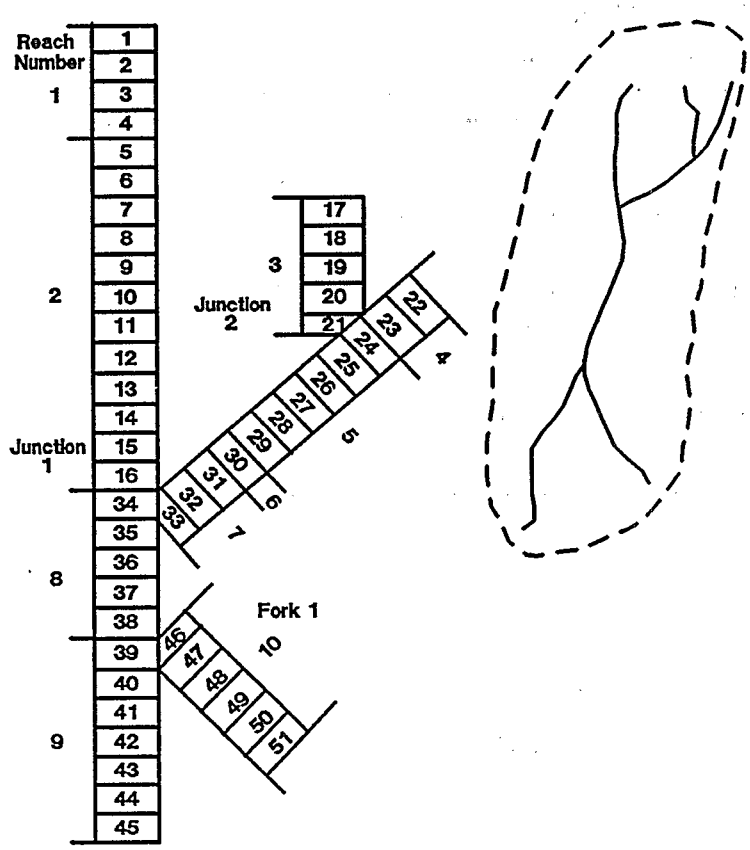

Figure 3. Stream network of computational elements and reaches.

TABLE 1. POINT SOURCE LOADS

	Reach									
	1	2	3	4	5	6	7	8	9	10
P.L., kg/day	0	1865	0	0	0	0	0	0	0	0
BOD, mg/L	2.19	21.58	1.97	18.78	5.26	0.69	0.71	9.79	9.31	9.14

TABLE 2. RESULTS

	Reach									
	1	2	3	4	5	6	7	8	9	10
C_*, mg/L	2.2	21.6	2.0	18.8	5.3	0.7	0.7	9.8	9.3	9.1
MPL kg/day	0	1870	0	0	0	0	0	0	0	0

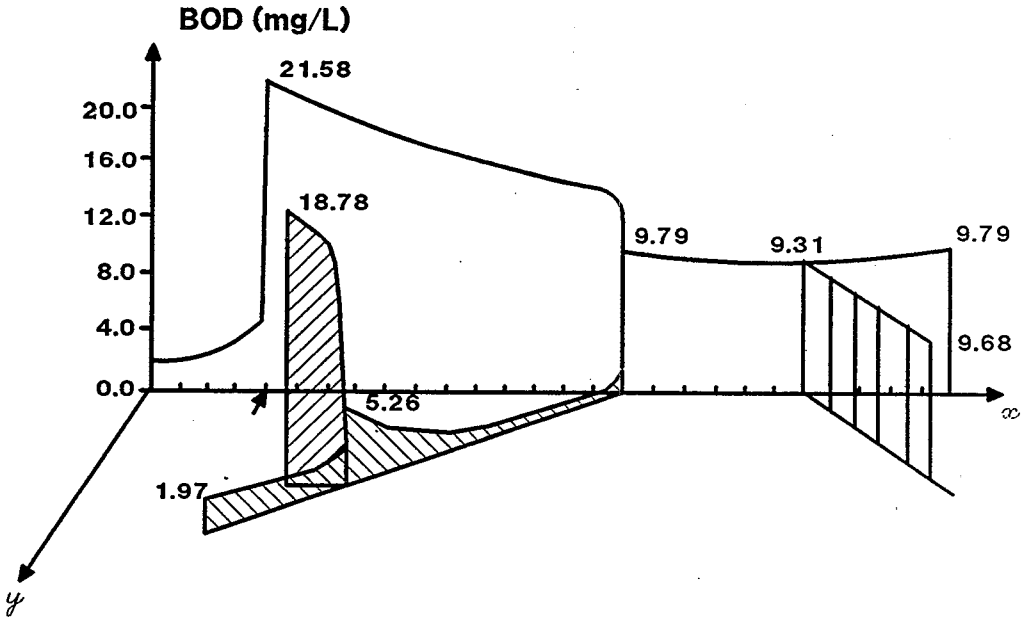

Figure 4. BOD simulation result.

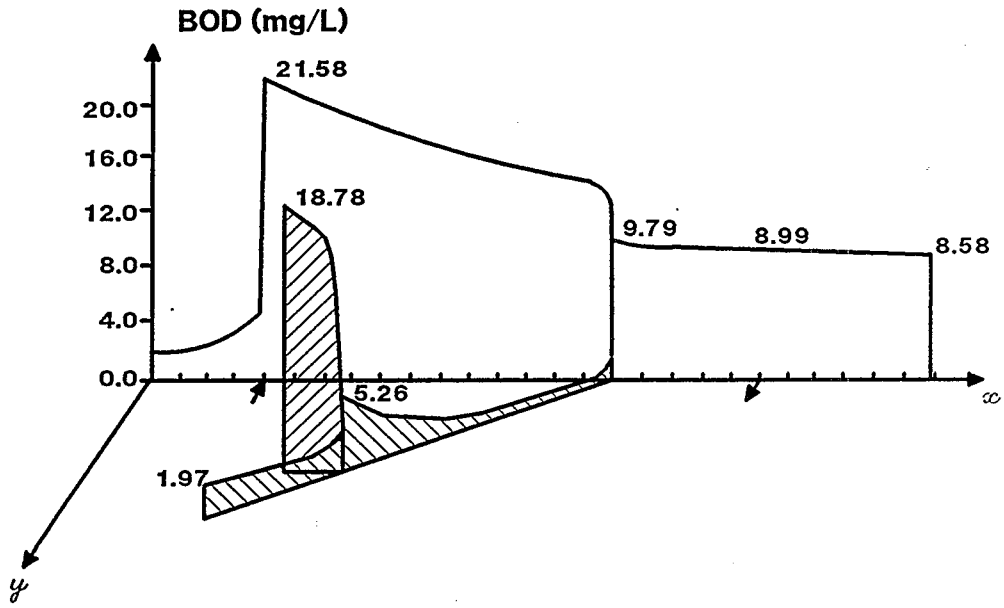

Figure 5. River system and BOD simulation results from EPA's QUAL2E workshop, Nanjing, 1985.

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