ONTOGENY OF NITROGEN METABOLISM

AND EXCRETION IN TELEOST FISHES

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Nitrogen metabolism and excretion during fish development is a critical aspect of early physiology because the major fuel source in most species is obtained endogenously through absorption of yolk proteins. Catabolism of proteins and amino acids results in the formation of ammonia, a potentially toxic nitrogenous end-product that must be either eliminated or modified to prevent damage to the developing embryo. Alternatively, if ammonia is sequestered or retained, this may occur in a compartment separate from the developing organs (eg., yolk sac). Nitrogen excretion during early life stages is not static but is influenced by developmental stage, environmental conditions, and in some cases, maternal factors.

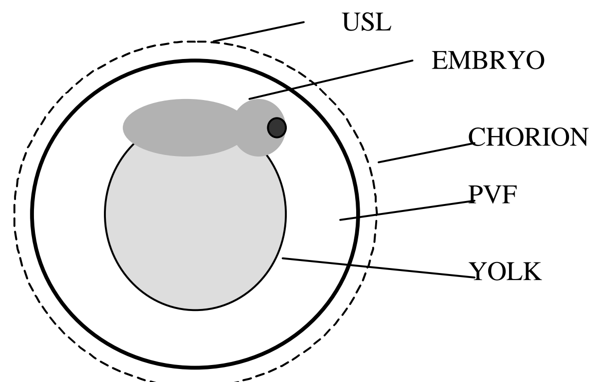
The physical properties of the embryo influence the rate of nitrogen elimination to the external environment. A typical teleost embryo is shown below, with the unstirred water boundary layer (USL), the developing embryo with the attached yolk sac, surrounded by the perivitelline fluid (PVF)
and chorion or egg capsule. The development of gas and ion exchange surfaces are of primary importance to the excretion of nitrogenous wastes. Nitrogen excretion in embryos occurs in the absence of functional gill filaments, and even after hatch, cutaneous gas exchange may initially dominate.

In rainbow trout embryos, ammonia excretion is dependent on the NH_3 partial pressure gradient (P_{NH3}) from the embryo to the water. (Rahaman-Noronha et al., 1996). There is an acidic USL that facilitates ammonia excretion by maintaining the P_{NH3} gradient through conversion of NH_3 to NH_4+ upon entry into the USL, as in adult trout. Urea excretion constitutes a significant portion of

total nitrogen excretion in some embryos. Diffusion of urea from the trout embryo was dependent, in part, on a bi-directional facilitated urea transporter with a Km of 2 mM (Pilley et al., 2000). Urea analogs (i.e., thiourea, acetamide) and inhibitors (i.e., phloretin, NPTU) added to the external water reversibly inhibited urea excretion from the embryo. The tissue localisation (eg., yolk sac membrane, cutaneous surface) of this putative urea transporter is unknown.

Ammonia is excreted soon after fertilization, but it also steadily accumulates, peaking before or just after hatching (e.g. Wright et al., 1995; Chadwick and Wright, 1999). Is ammonia a trigger for hatching? Several environmental factors have been implicated in the hatching process, including oxygen availability, alkaline water and temperature. We tested the hypothesis that ammonia is a trigger for hatching in rainbow trout embryos. Eyed-up embryos (28 days post fertilization (dpf), 10° C) were exposed to external ammonia for 2 h (10 mM NH_4Cl) or 4 days (0.2 mM NH_4Cl), treatments that elevated internal ammonia concentrations. Elevated external ammonia, however, did not significantly effect the time to hatch, possibly because of efficient mechanisms that maintained low ammonia levels (see below).

In further studies we examined potential pathways for ammonia detoxification. It appears that urea is synthesized early in embryogenesis, because urea accumulates and/or it is excreted to the water. Urea cycle enzymes (carbamoyl phosphate synthetase (CPSase III), ornithine carbamoyl transferase (OCTase), arginase, and glutamine synthetase (GSase)) are induced in whole embryos of the freshwater rainbow trout (Wright et al., 1995; Korte et al., 1997), marine Atlantic cod (Chadwick and Wright, 1999) and Atlantic halibut (Terjesen et al.,



1999). Recent work on a variety of teleost species has demonstrated that CPSase

III and other urea cycle enzymes may be located primarily in extra-hepatic tissues of adult fish (Felskie et al., 1997; Kong et al., 1998). Hence, we investigated the tissue location of urea cycle enzymes in rainbow trout embryos. Just-hatched embryos (38 dpf) were separated into three fractions using a dissecting microscope; the yolk, the liver, and the embryonic body. There was no CPSase III activity in yolk or liver tissue, but significant levels of activity were detected in the embryonic body. GSase, OTCase and arginase activities were present in yolk, liver and the embryonic body, but the highest activities were extra-hepatic (embryonic body).

In addition to urea synthesis, another possible pathway for ammonia detoxification is via the formation of nonessential amino acids, particularly glutamine (Gln) and glutamate (Glu). To examine the mechanisms of ammonia detoxification, trout embryos were exposed to environmental conditions that impaired ammonia elimination, that is alkaline water (pH 9.5; 12 C, hatched embryos, 31 dpf) or elevated ammonia (0.2 or 10 mM NH_4Cl, 10 C) for 2 h or 4 days (embryos, 28 dpf). These environmental perturbations resulted in decreased rates of ammonia excretion and increased rates of urea excretion. There was no significant differences in either whole embryo Gln or Glu concentrations in ammonia-exposed relative to control fish. Embryonic tissue was separated from yolk using a new centrifugation technique recently developed in our group (A. Shahsahavarani, J. Ballantyne, and P. Wright, in preparation). By separating embryo from yolk, we can more carefully monitor changes in metabolite levels in the tissues which may be quite different from changes that occur in the yolk. Following acute or chronic exposure to NH_4Cl, yolk ammonia levels increased by 2- to 4-fold but surprisingly, tissue levels were unchanged. At the same time tissue urea levels were significantly higher suggesting that excess ammonia was converted to urea as a detoxification mechanism. Urea cycle enzyme activities were not significantly different between the control and treated embryos. Thus, the level of activity of the urea cycle enzymes may have been sufficient to meet the increased flux through the pathway, or possibly urea was produced via another pathway, such as uricolysis.

Taken together, the data indicate that trout embryos are very capable of handling excess ammonia. Despite exposure to external ammonia for up to 4 days, there were no changes in the time to hatch compared to control embryos. Embryos maintain low tissue ammonia levels despite an inwardly directed (water-to-embryo) ammonia gradient. This may be achieved, in part, by sequestration of ammonia in the acidic yolk and detoxification by conversion to urea. Sensitivity to elevated environmental ammonia in rainbow trout is reported to be lower in

embryos relative to later stages of development. The results reported here indicate that trout embryos have efficient mechanisms to cope with excess ammonia and this may help to explain their relatively high tolerance of external ammonia.

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URICOLYSIS AND THE ORNITHINE-UREA CYCLE

DURING ONTOGENY

OF THE AFRICAN CATFISH, *CLARIAS GARIEPINUS*

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Due to the high degree of utilisation of amino acids for energy and assumed absence of specialised excretory organs for voiding ammonia, urea may be an alternative vehicle for nitrogen excretion or storage during early teleost development. In investigations of the facultative air-breathing African catfish *Clarias gariepinus* (Burchell 1822), we found that around metamorphosis as much as 44% of the total nitrogen excretion was due to urea-N (Terjesen *et al*., 1997). This species is tolerant to variations in water availability, temperature and salinity. We consequently studied the pathways for urea production in this species. Although the ornithine-urea cycle (OUC) in embryonic teleosts has received increasing attention the last few years (Wright *et al*., 1995; Chadwick and Wright, 1999; Terjesen *et al*., 2000), little is known about uricolysis.

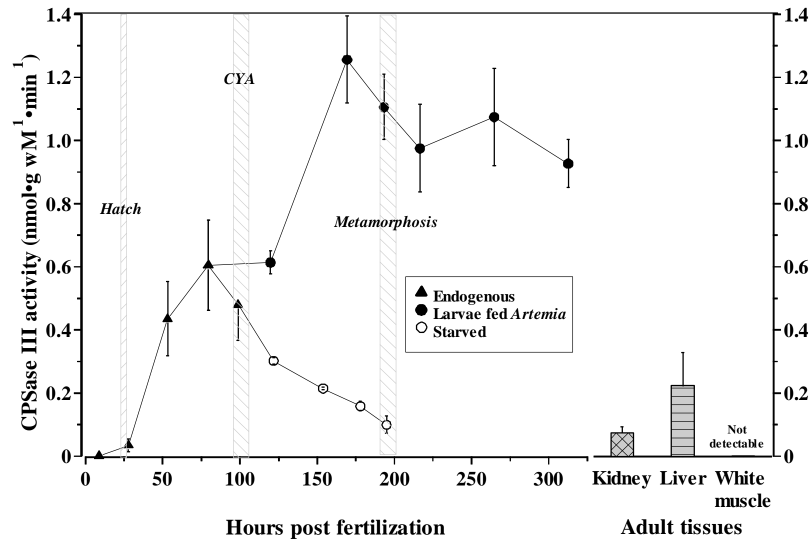

Figure 1. Carbamoyl phosphate synthetase III (CPSase III) activity during ontogeny of *Clarias gariepinus*. Each datapoint refers to the average (±SD) of three samples, except at 79 and 120

Rearing and sampling procedures were conducted after Terjesen *et al*. (1997). The OUC was analysed after Chadwick and Wright (1999) and Terjesen *et al.* (2000). Uricase was measured with a continuous assay at 293 nm. Allantoinase activity was estimated by an end-point assay employing differential analysis of glyoxylate derivatives. Allantoicase and ureidoglycollate lyase (UGL) actvity were estimated with continuous assays employing LDH and NADH and followed at 340 nm.

The key regulatory OUC enzyme carbamoyl phosphate synthetase (CPSase) III was detected throughout development of *C. gariepinus* (Fig. 1). Arginase, ornithine carbamoyl transferase, and glutamine synthetase were also detected (data not shown). Argininosuccinate synthetase and lyase were not measured but are generally found in teleost tissues. The OUC appears therefore to be expressed in embryos and larvae of *C. gariepinus*. Adult *C. gariepinus* expressed only low levels of CPSase III, which was surprising since *C.*

batrachus express significant levels of CPSase III, even when not challenged by water deprivation or ammonia exposure (Saha and Ratha, 1989). It is possible that in adult *C. gariepinus*, the OUC is only expressed during harsh environmental conditions.

Unlike the CPSase III of larval Atlantic halibut (Terjesen *et al*., 2000), *C. gariepinus* CPSase III showed virtually no activity without its positive effector *N*-acetyl-L-glutamate (AGA) present in the reaction mix, even at high glutamine concentrations. We suggest that this enable the OUC to be more efficiently controlled by AGA in face of varying glutamine concentrations, in contrast to the Atlantic halibut. Experiments were also conducted where *C. gariepinus* larvae were dissected for liver and tail (*i.e.* muscle) pieces. These samples were subjected to gel filtration chromatography. The muscle elution showed two peaks with CPSase activity, characterised to be CPSase II and III, while the liver elution was devoid of CPSase activity. This suggest that CPSase III is only expressed in muscle in larval *C. gariepinus*. By extending the suggestion by Lindley *et al*. (1999), it may be that the liver is too small for accommodating a substantial increase in expression during periods of adverse environmental conditions.

C. garipinus larvae expressed high levels of CPSase III compared to other studied larval teleosts, but magnitudes lower than in elasmobranchs and batrachoid fishes. Furthermore, by comparing total urea production rates of *C. gariepinus* larvae with the CPSase III activity, it was found that the OUC at no stage in development could account for more than 20% of the produced urea. Hydrolysis of arginine by arginase can account for an aditional 65%. Consequently, additional urea producing system(s) must be present. All four uricolytic enzymes were detected in embryos and larvae as well as in adult liver (Fig. 2: uricase). Uricase appears to be the rate limiting step of uricolysis. Starved larvae showed the highest activity, possibly because of increased cell degradation liberating nucleotides for breakdown. UGL showed considerable activity, suggesting that urea production is not limited to the allantoicase reaction but is a two-enzyme process in which UGL splits of a second urea molecule. Uricolysis could account for all urea produced. However, the present study reports the maximum *in vitro* capacity for urea production through the pathways, and clearly, *in vivo* experiments employing radiotracers should be conducted to confirm these findings.

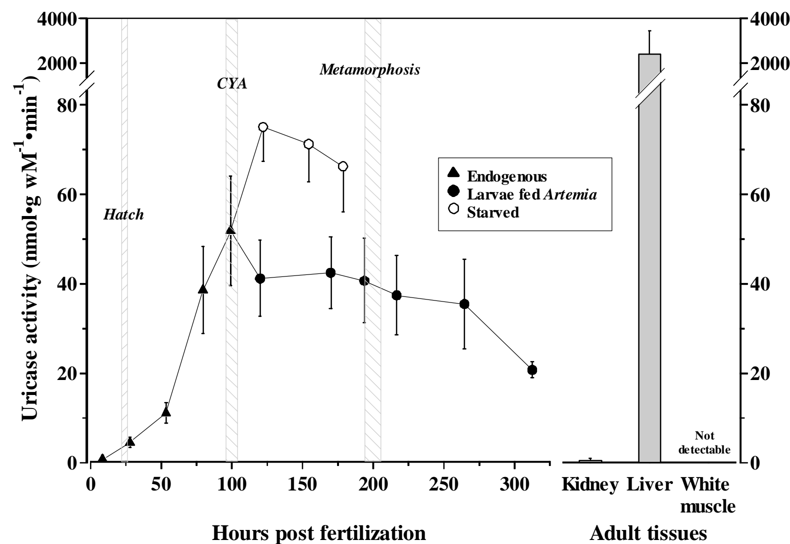

Figure 2. Uricase activity during ontogeny of *Clarias gariepinus*. Each datapoint refers to the average (±SD) of 3-6 samples. wM= wet mass; CYA = complete yolk

In conclusion, the observation that the maximum *in vitro* capacity of CPSase III is below that of urea production, suggests that other pathways predominate during early development. Possibly, the OUC is expressed at higher levels, and is quantitatively more important during periods of adverse environmental conditions.

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THE MATERNAL-FETAL RELATIONSHIP IN THE EELPOUT *ZOARCES VIVIPARUS*: IMPACTS FROM THE ENVIRONMENT

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Abstract

Pregnant females of the eelpout *Zoarces viviparus* were exposed to different environmental factors to investigate the impact on metabolites which may elucidate physiological interrelations between the embryos and the pregnant motherfish. The maternal organism was exposed to different salinities, elevated levels of nitrogenous endproducts and to different concentrations of the xenobiotic compound octylphenol, respectively. The concentrations of ammonia, urea, low molecular compounds, chloride, and calcium were measured in the maternal plasma and the ovarian fluid, the ambient medium of the embryos. Ammonia increased in the maternal plasma as well as in the ovarian fluid by the highest salinity (28ppt). No change could be observed in the level of urea or chloride by increased salinities. By exposing the embryos to increased levels of urea and ammonia in their ambient medium, the ovarian fluid, it was observed that a steady flow of the nitrogenous compounds occurred from the ovarian fluid to the maternal plasma. In the fish exposed to octylphenol the calcium concentration in the ovarian fluid decreased in a dose-dependent manner by increasing concentrations of the estrogenic compounds. In contrast significant increased levels of total calcium were observed in the plasma of the exposed motherfish.

Introduction

The eelpout *Zoarces viviparus* is an euryhaline viviparous teleost living in near-coastal waters of the North Sea. The viviparous female fish has a complex pattern of reproductive activity. Fertilization follows a summer period of

intensive final vitellogenesis and the subsequent gestation occurs in the ovarian lumen of a single ovary. The embryonic development, lasting approximately 5 months, may be divided in three different stages: a prehatch stage, a posthatch yolksac stage followed by a lengthly post-yolksac developmental stage. The last two stages are characterized by an intensive maternal-fetal trophic relationship (Korsgaard and Andersen, 1985; Korsgaard, 1986). The ovarian fluid constitutes the ambient environment of the embryos in the ovary. This fluid is shown to undergo rapid turnover of metabolites. Studies with radiolabeled compounds indicate that the embryos obtain energy from catabolism of low molecular metabolites such as free amino acids (Korsgaard, 1992). As the embryos constitute a very dense population within the ovarian lumen, their survival and growth are dependent on the maternal capability to provide nutrients and oxygen sufficiently and to remove waste and potential toxic products effectively (Korsgaard *et al.* 1995) as well as securing a steady osmotic environment. Environmental impacts on the maternal-fetal relationship *in vivo* were investigated by exposing the fish to elevated levels of urea and ammonia, to different salinities and to the xenobiotic compound octylphenol measuring the subsequent time-course changes in the level of different ions, metabolites and nitrogenous endproducts in the maternal plasma and the ovarian fluid, the latter constituting the ambient embryonic medium.

Material and methods

In the salinity experiment pregnant fish were exposed to 5, 15 and 28 ppt seawater and samples were taken of plasma and ovarian fluid after 24 h.
In the experiment elucidating the effect of elevated levels of urea and ammonia, urea (500µmol urea-N/100g BW) and ammonia (30µmol ammonia-N/100g BW) were injected into the ovarian fluid and the level of the two nitrogenous components were followed during a 24 h time-course.
In the xenobiotic experiment pregnant fish were exposed to different concentrations, 25 and 100 µg/L of 4-t-octylphenol in the ambient seawater using an estrogen-exposed (0.5 µg/L) and a control group, respectively, as reference groups. The fish were sampled after an exposure period of 5 weeks.

Results and Discussion

No significant changes were observed in the chloride concentration in the plasma or ovarian fluid, respectively, in the different salinity groups. However, the chloride concentrations were observed at a lower level in the ovarian fluid when compared to the plasma levels at all three salinities (Figure 1).

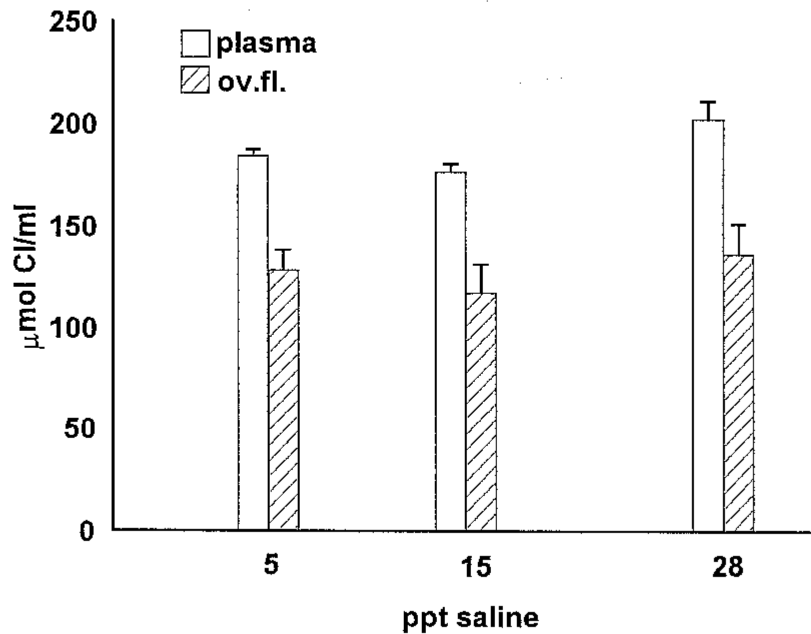

Fig. 1. The concentration of chloride in maternal plasma and ovarian fluid at three different salinities after 24 h acclimation.

Only a few data exist regarding salinity tolerance in viviparous fish. In newborn guppies (*Poecilia reticulata*) the salinity tolerance increased within 5 days after birth concomitant with an increase in size of the chloride cells (Shikano and Fujio,1999). The present study indicates that the embryos are protected from increased levels of chloride by the constant low levels of chloride in the ovarian fluid. No increase could be observed in the concentration of urea in the ovarian fluid or in the plasma when comparing the fish by the different salinities.

In the guppy *Poecilia reticulata* urea was found to be increased in embryos during later development indicating a role of urea as an osmotic component during development in a viviparous fish (Depeche, 1975).

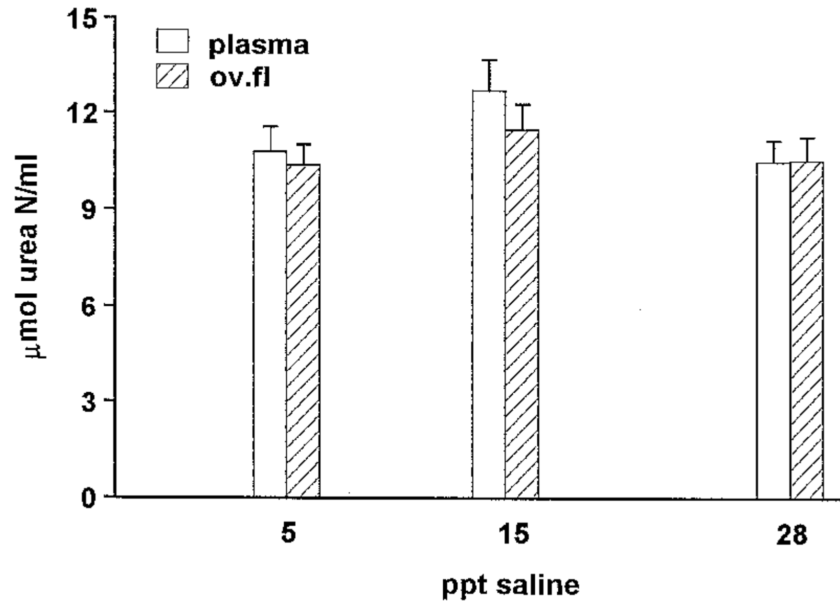

Fig. 2. The concentration of urea in the maternal plasma and ovarian fluid at 3 different salinities after 24 h acclimation.

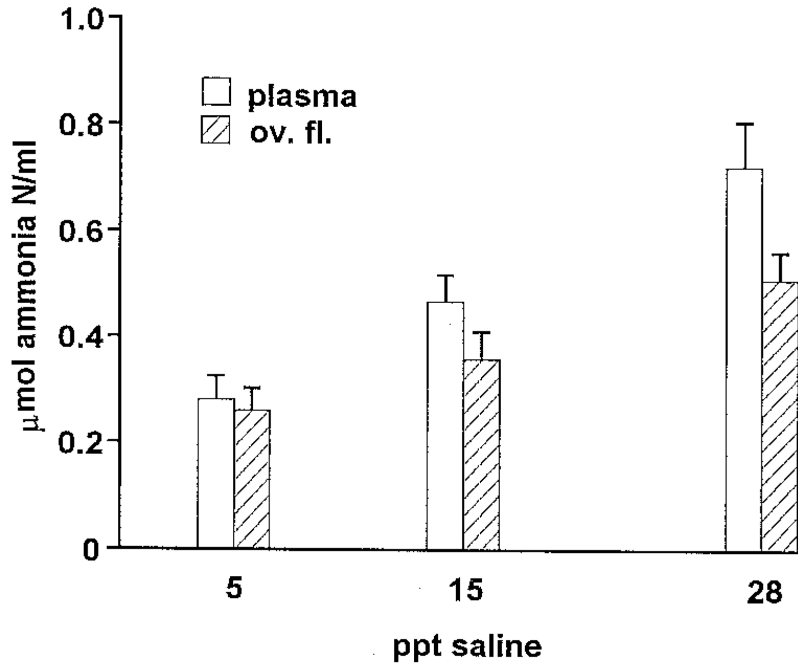

Fig. 3. The concentration of ammonia in maternal plasma and ovarian fluid at three different salinities after 24 h.

The concentrations of ammonia were increased in the plasma as well as in the ovarian fluid by increased salinities (Fig. 3). The results from the present study indicate that urea is not an osmoregulatory component in the maternal-fetal relationship. Time-course studies, however, are needed to elucidate the role of urea and ammonia in relation to increased salinities.

Figure 4 depicts a steady decrease in urea in the ovarian fluid during the 24 h time-course after an intraovarian loading and a concomitant increase in the plasma level. A similar pattern occurred by loading with ammonia.

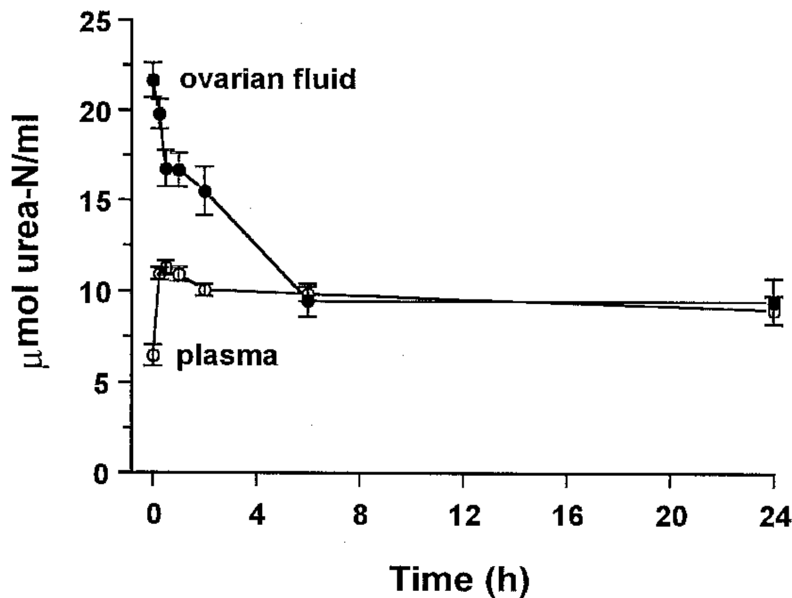

Fig. 4. Time-course levels of urea in the maternal plasma and ovarian fluid after an intraovarian loading with urea at 0 h.

The results indicate a steady flow of nitrogenous compounds from the pregnant ovary into the maternal circulation, a system which may protect the embryos from being exposed to high levels of the nitrogenous end products under the natural crowded conditions in the pregnant ovary (Korsgaard *et al.* 1995).

In the experiment elucidating the effect of the estrogenic compound octylphenol no significant changes could be observed in the concentration of chloride in the ovarian fluid or of ammonia and urea in either of the exposed groups when compared to the controls. In this experiment the concentration of calcium was measured, as calcium is a crucial component for the synthesis of the yolk-precursor protein vitellogenin, which is induced by xenoestrogenic compounds. That vitellogenin was synthesized by the motherfish as a result of the estrogenic exposure by octylphenol and estradiol was confirmed by significantly increased levels of circulating vitellogenin measured by ELISA. Interestingly a significant effect of the exposure could be observed in the concentration of total calcium in both plasma and ovarian fluid as shown in Table 1.

Table 1. The effect of exposure to xenoestrogens on metabolites in plasma and ovarian fluid of pregnant eelpout.

	Urea mol N/ml	Calcium mg/100ml	Glucose mg/100ml	NPS mg/100ml
Plasma				
Control	4.65 (0.36)	11.03 (0.50)	26.66 (1.56)	15.5 (0.60)
OP25	5.28 (0.69)	27.43 (3.06)**	26.45(1.34)	13.6 (0.10)
OP100	3.26 (0.88)	61.44 (1.35)**	13.46 (3.13)*	9.4 (0.55)**
Estradiol	5.08 (0.25)	52.65 (6.48)**	28.77 (4.51)	7.3 (0.11)**
Ov. Fluid				
Control	5.18(0.45)	7.7(0.3)	7.0(0.9)	0.08(0.007)
OP25	5.41(0.33)	5.5(0.8)	9.3(0.7)	0.07(0.005)
OP100	4.27(0.36)	2.3(0.28)**	10.4(1.3)	0.29(0.18)
Estradiol	6.46(0.33)	1.8(0.35)**	10.4(1.5)	0.11(0.02)

Mean ± SEM * P<0.001 compared to controls **P<0.0001 N=7-8

The concentration of calcium in the ovarian fluid decreased in a dose-dependent manner in the octylphenol exposed groups and was also observed to be significantly decreased in the ovarian fluid of the pregnant fish exposed to estradiol, when compared to the control level. In contrast the corresponding level of calcium in the plasma showed a marked increase in all treated groups, most likely due to the increase in vitellogenin-bound calcium. The observed decrease in the level of calcium in the ovarian fluid as well as the decreased level of amino acids (NPS) in the maternal plasma may indicate that these

compunds are mobilized in increasing amounts for the hepatic synthesis of vitellogenin in the exposed fish. This may occur at the cost of the normal embryonic development. Thus a negative impact on the calcification process was indicated by deformities in the embryonic skeleton observed in the embryos from motherfish exposed to the estrogenic compound.

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THE EARLY LIFE HISTORY

OF AN ANCIENT VERTEBRATE:

METABOLIC WASTE PRODUCTION

IN THE SEA LAMPREY (*PETROMYZON MARINUS*).

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Introduction

As with all lampreys, the sea lamprey (*Petromyzon marinus*) has a prolonged larval stage in which the animals remain burrowed in the substrate of freshwater streams as suspension feeding ammocoetes for 3-7 years. Following the larval phase, the ammocoetes cease feeding and enter a complex metamorphosis characterized by elevated metabolism and major structural changes in the gills, liver, gastrointestinal tract, kidneys and feeding apparatus (Youson, 1997). These changes prepare sea lampreys for the parasitic phase of their life cycle, when they feed on the blood of fishes, but little is known about how patterns of nitrogenous waste metabolism reflect the life history of these animals. Accordingly, the goal of the present study was to test the hypothesis that patterns of nitrogenous waste metabolism and excretion reflect the sea lamprey's life history stage. As most of the sea lamprey's life cycle is spent in the burrow dwelling larval phase, this early life history stage was the main focus of this study.

Methods and Materials

Experimental Animals and Set-Up:

Larval sea lampreys (*Petromyzon marinus*), weighing 2-3 g, were collected from freshwater streams using pulsed D.C. electrofishing and housed in 200-500 L aquaria, which were also filled with silica sand (depth ~ 5cm) to serve as burrowing substrate. The patterns of nitrogenous waste (N-waste) metabolism in larval lampreys were then compared to sub-sets of post-metamorphic lamprey (2-3 g), parasitic (10-65 g), or adult (spawning; 100-300g) lampreys. The parasitic lampreys completed metamorphosis in the lab or were captured in Lake Huron and shipped to the laboratory where they were given access to rainbow trout (*Oncorhynchus mykiss*) as food in a 500 L Living Stream. Spawning sea lampreys were captured during their upstream spawning migration and transported to the laboratory, where they were held in large (200 L) flow-through aquaria. Holding conditions and experimental water temperatures were maintained between 10 and 15°C for all animals.

Experimental Protocol:

Initially, the basal levels of ammonia and urea excretion were monitored in sea lampreys belonging to each life stage (larval, parasitic, spawning) by taking 10 mL water samples from the closed flux chambers at regular time intervals. Changes in water ammonia and urea concentration were then used to determine the respective rates of ammonia (J^{Amm}) and urea (J^{Urea}) excretion (see Wilkie et al. 1999 for further details). In larval and parasitic lampreys, the animals were denied access to food for a minimum of 1 week to minimize the effects that feeding could have on patterns of N-waste metabolism; spawning lampreys do not feed.

Following each experiment, each group of animals were anaesthetized, and liver, intestine and muscle samples were taken from each animal and quickly frozen in liquid nitrogen using stainless steel tongs. The activities of key enzymes associated with amino acid catabolism (glutamate dehydrogenase, transaminase enzymes) were then determined on each set of tissues. Western blot analysis was also performed to quantify the levels of GDH present at each life stage.

Finally, based on observations that there are major structural changes to the gills and kidneys following metamorphosis, the role of the gill as an organ of nitrogenous waste excretion was assessed in the larval lampreys. This was initially done by taking advantage of the larval lamprey's ability to readily tolerate air exposure for several days (Potter et al. 1996), which would eliminate the gills as an excretion site. By air exposing the larval lampreys for 3 d, and monitoring ammonia and urea excretion patterns and internal stores, we were able to determine if excretion was impeded during emmersion. Experiments employing miniature divided flux chambers where then used to quantify the relative contribution that the gills and extra-branchial sites (skin + kidneys) made to nitrogenous waste excretion in this early life history stage. These data were then compared to similar data generated in post-metamorphic lampreys, and spawning lampreys fitted with indwelling renal catheters.

Results and Discussion

Compared to later life stages, rates of N-waste excretion ($J^{N\text{-}waste}$) were very low in larval lampreys, as J^{Amm} was approximately 25 $nmol g^{-1} \cdot h^{-1}$ and J^{Urea} about 15 $nmol g^{-1} h^{-1}$ (Figure 1). Overall, $J^{N\text{-}waste}$ was about 80 percent lower than in comparably sized post-metamorphic and parasitic lampreys (Figure 1). These findings suggested that larval lampreys have a relatively low amino acid deamination capacity. This interpretation was further supported by the low activities of glutamate dehydrogenase and the transaminase enzymes found in the liver, muscle and intestine of larval lampreys (Figure 2). Western Blot analyses indicated that the quantity of glutamate dehydrogenase was also substantially lower in larval lampreys (Figure 2). The larval lamprey’s low amino acid deamination capacity may be explained by their low metabolic rates and diet, which is mainly comprised of detritus (Sutton and Bowen 1994). Conversely, the greater amino acid deamination capacity of parasitic phase lampreys likely benefits these animals when they are at a life history stage where they will be ingesting high amounts of protein rich blood.

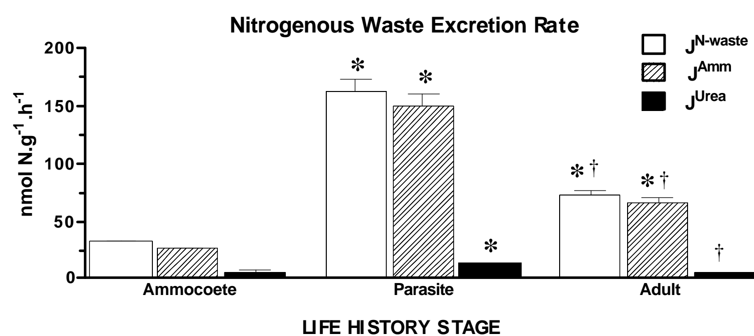

Figure 1. Total nitrogenous waste excretion rates ($J^{N\text{-waste}} = J^{Amm} + J^{Urea}$; open bars), ammonia excretion rates (J^{Amm}), and urea excretion rates (J^{Urea}) in larval (ammocoetes), parasitic and adult sea lampreys. Asterisks represent significant differences from larval lampreys, while daggers symbolize significant differences from parasitic lampreys ($P \leq 0.05$).

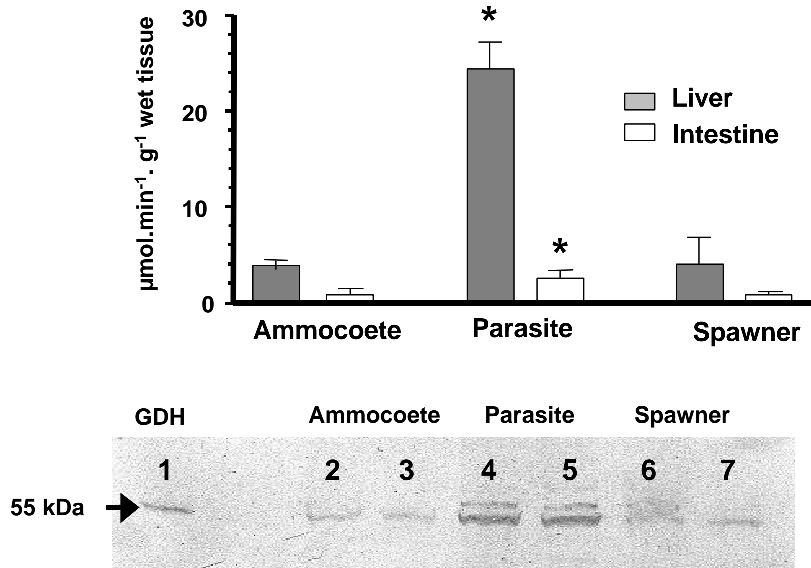

Figure 2**. Top Panel:** Glutamate dehydrogenase (GDH) activities in the liver (solid bars) and intestine (open bars) of larval (ammocoetes), parasitic and spawning sea lampreys. **Bottom Panel:** Representative Western Blot of GDH levels in the livers of ammocoetes (lanes 2,3), parasitic lampreys (lanes 4,5) and spawning sea lampreys (lanes 6,7). Asterisks represent significant differences from larval and spawning lampreys ($P \leq 0.05$).

In addition to low rates of N-waste excretion, the very different body structure and habitat of larval lampreys could also be reflected by differences in the mechanisms used to excrete N-waste. To determine how important extra-branchial routes of N-waste excretion were in larval lampreys, a sub-set of animals were emmersed for 72 h. As expected, the larval lampreys readily survived out of water during emmersion, and following subsequent re-immersion, with no changes in whole body water content or haematocrit. Although there were slight reductions in J^{Amm} and J^{Urea} during the first 8-12 h of air exposure, $J^{N\text{-}waste}$ was fully re-established by 24 h (Figure 3). This recovery, and the absence of any build-ups of plasma ammonia or urea during air exposure, indicated that the larval lampreys were likely using extra-branchial routes of excretion. This finding was confirmed using the divided flux chamber apparatus, which indicated that only 30 percent of the larval lamprey's N-waste were excreted via the gills, while 65 percent was excreted using extra-branchial routes such as the kidneys, and perhaps the skin (Figure 4A). Since larval lamprey skin is not well vascularized, excretion via this route was likely minimal. Ammonia excretion was equally distributed between branchial and extra-branchial routes, but more than 85 percent of urea was excreted via the latter pathways (Figure 4A).

Following metamorphosis, the gills became the predominate N-waste excretion site, accounting for more than 80 percent of total N-waste excreted by post-metamorphic and spawning lampreys (Figures 4B, 4C), which is the case in most freshwater fishes (Wood 1993). Interestingly, the predominate site of urea excretion continued to be extra-branchial routes in the post-metamorphic lampreys, and virtually all urea was excreted via renal routes in spawning lampreys, fitted with indwelling renal catheters (Figures 4B, 4C).

A greater reliance on extra-branchial routes of excretion in larval lampreys would suit their burrow dwelling habits. In general, larval lampreys are found burrowed in the silty substrate of streams (Young et al. 1990), where ammonia may accumulate due to the microbial degradation of organic matter (Sarda and Allen-Burton 1995). A lower dependence on branchial routes of excretion would therefore make larval lampreys less vulnerable to fluctuations in environmental ammonia because they would not have to rely on the presence of a blood-water ammonia diffusion gradient to promote ammonia excretion. As a result, larval lampreys could continue to excrete ammonia even when environmental ammonia concentrations were elevated. The larval lamprey's ability to excrete urea and its very high tolerance to environmental ammonia would also suit their burrow dwelling life style (Wilkie et al. 1999).

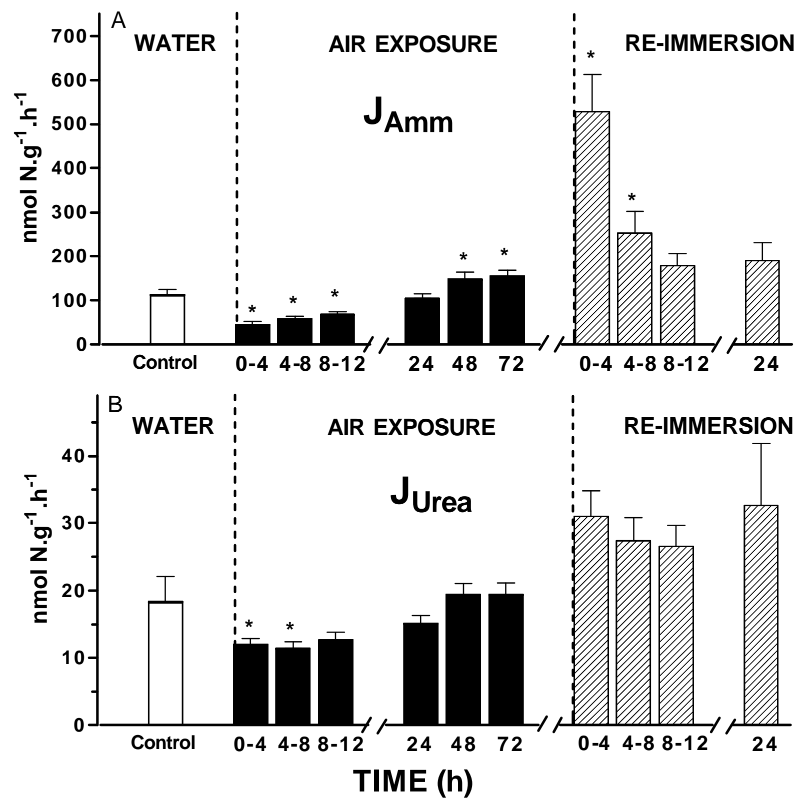

Figure 3**.** (A) Ammonia excretion rates (J^{Amm}), and (B) urea excretion rates (J^{Urea}) in larval lampreys (ammocoetes) during 72 hour of air exposure. Asterisks represent significant differences from control excretion rates in water ($P \leq 0.05$).

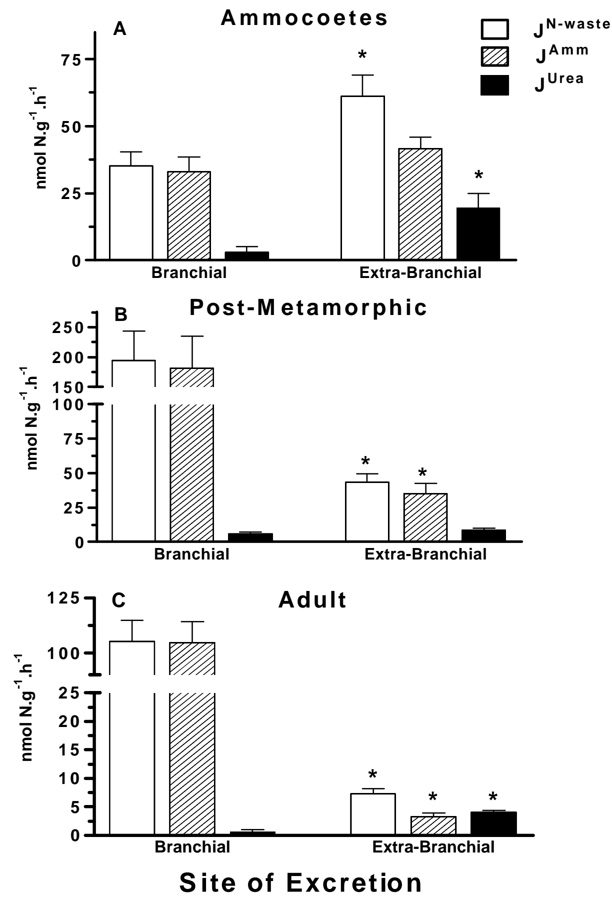

Figure 4. Routes of total nitrogenous waste excretion ($J^{N\text{-waste}}$; open bars), ammonia excretion (J^{Amm}; hatched bars), and urea excretion (J^{Urea}; solid bars) in (A) larval (ammocoetes), (B) post-metamorphic, and (C) adult (spawning) sea lampreys. Data are expressed as the rate of excretion measured at the gills (branchial) or extra-branchial sites (kidneys + skin). Asterisks indicate significant differences between branchial and extra-branchial routes ($P \leq 0.05$).

Summary and Conclusions

Compared to later life history stages, the prolonged larval stage of sea lampreys is characterized by very low rates of N-waste excretion, low activities of key amino acid catabolizing enzymes and secondary reliance on the gill as a route of ammonia or urea excretion. We conclude that these differences reflect the vastly different habitat, life style, body structure and diet of larval lampreys as compared to the lamprey's later life history stages.

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THE DEVELOPMENT OF THE THYROID GLAND IN TELEOSTEAN EMBRYOS: HISTORICAL PERSPECTIVES

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Abstract

The paper briefly reviews historical and recent work dealing with the early ontogeny of the thyroid primordium in teleostean fish embryos, with particular emphasis on salmonid species. The recent work describing the appearance of the thyroid primordium as a tubular structure, the bifurcation of the primordium at its anterior and posterior margins to form a complex scaffold-like complex is described, as is the delay in the appearance of thyroid follicles , becoming evident only in the late embryo or early juvenile stages. Also, methods that allow the determination of the time of onset of synthesis and secretion of thyroid hormones by embryos are examined, as is the evidence of the first presence of thyroid hormone receptors prior to the appearance of the thyroid tissue in the embryo, and the impact of treatment of embryos with exogenous thyroid hormone, or exposure of the embryos via yolk thyroid hormones of maternal origin on developmental events.

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*Presenting author

Introduction

The complex interrelated events associated with the early developmental biology of vertebrates represent one of the most challenging research areas in biology, and during the last decade, there has been an increasing interest in understanding the factors, including the endocrine factors, that regulate early developmental biology. The thyroid hormones (TH s) are known to play an essential role in early vertebrate ontogeny, and are particularly important for the normal development of the central and peripheral nervous system, heart, liver, intestinal tract and skeletal and smooth muscle (reviewed by Hsu and Brent, 1999). Work in teleost fishes has been less intense, although ongoing work in several laboratories is examining the expression of T_3-receptor (TR) genes during early development (e.g., Yamano and Miwa, 1998; Llewellyn et al., 1999), and attempting to determine the timing of the functional maturation of the hypothalamus-pituitary gland-thyroid tissue (HPT) axis (Raine and Leatherland, 1999, 2000). Of particular interest in this regard is consideration of the role played by the thyroid hormones that are present in the yolk of fish eggs and embryos. This source of hormone represent a relatively uncontrolled exposure of the embryo to thyroid hormone during critical developmental periods (reviewed by Leatherland, 1994), and the significance of the yolk hormones is still not well understood.

In this paper, we review briefly early and current work dealing with the role of thyroid hormones in early development of teleostean fishes, with particular emphasis on trout and salmon species. Of special emphasis is the early ontogeny of the thyroid tissue, the timing of the onset of thyroid hormone synthesis and secretion by the embryo, the effect of exogenous and maternal sources of TH on the early development of the embryo, and the timing of the appearance of TR's in the embryo.

Early ontogeny of thyroid tissue in fish embryos

Since the late 19th Century (Maurer, 1886), there have been only 3 papers published that deal with the early ontogeny of the thyroid tissue of teleost fishes [Atlantic salmon, *Salmo salar* (Hoar, 1939), fathead minnow, *Pimephales promelas* (Wabuke-Bunoti and Firling, 1983), and rainbow trout, *Oncorhynchus mykiss* (Raine and Leatherland, 2000)]. In fact, there has been only a handful of studies related to thyroid ontogeny in any of the vertebrate classes. There is

general agreement that the thyroid primordium originates as an evagination of the ventral pharyngeal floor, but there is no consensus as to how the thyroid follicles form. In the most recent work on rainbow trout, serial sectioning revealed that the primordium develops into a tubular structure and distinct follicles do not appear until relatively late in embryogenesis (40 days post-fertilization in trout raised at 8°C). When they are formed, the follicles are produced by the pinching off of bifurcated anterior and posterior branches of the tubular primordium. Even in juvenile trout, when the yolk has been fully absorbed, a tubular thyroid structure is present in addition to the follicles (J.C. Raine, A. Takemura and J.F. Leatherland, unpublished data). Thus the assessment of thyroid function in fish embryos and early juveniles, based solely on the measurements of thyroid tissue histological characteristics, such as the number of follicles, might be inappropriate.

Timing of the onset of thyroid hormone synthesis and secretion

The formation of the THs (thyrogenesis) involves the synthesis of thyroglobulin by the thyrocytes, the exocytosis of this protein into the follicular lumen of the thyroid tissue, and the extracellular iodination, oxidation and glycosylation of the protein, within the lumen. The release of the hormones involves pinocytosis of thyroglobulin by the thyrocytes and the proteolytic release, within cytoplasmic vesicles, of iodinated thyronine compounds that comprise elements of thyroglobulin structure. Because of the complexity of thyrogenesis and TH release or secretion, details of these events have only recently been described in the adult mammal thyroid gland, and relatively little is known of the processes in vertebrate embryos. There are very few studies of these events in fish embryos.

The timing of the onset of hormone production in fish embryos has been difficult to assess, particularly during the period before histologically distinct thyroid tissue is seen. The first incorporation of radioiodide into the embryo has been used by some authors as an indication of thyrogenesis (e.g., Greenblatt et al., 1989), although evidence suggests that much of this iodide becomes associated with the yolk, and not with the pharyngeal thyroid tissue (see discussion in Raine and Leatherland, 2000). Recently (Raine and Leatherland, 1999, 2000), we took a different approach to the problem, using immunohistochemistry to examine the period of thyroid development from first appearance of a primordium until the formation of follicular thyroid tissue in rainbow trout. We found temporal changes in the distribution of immunostaining

(IS) of the thyroid tissue with polyclonal antibodies raised against L-thyroxine (T_4) and triiodo-L-thyronine (T_3), and noted that IS was first seen at the periphery of the tubular primordium/follicle lumen. We proposed that this IS response was probably associated with the oxidative iodination of the thyroglobulin and secondary oxidation to form T_4 and T_3 that continue to be covalently bound within the thyroglobulin molecule. A second type of IS staining pattern was seen in embryos sampled a few days later, when IS was found both at the periphery of the follicles and also in association with some of the thyrocytes. The presence of and IS response in the thyrocytes was thought to represent thyroglobulin vesicles in which proteolysis of partial exposure of the THs was present for ligation with antibodies. These stages of thyroid function in rainbow trout embryos were referred to as Phases 1 and 2, respectively (Raine and Leatherland, 2000), and provide the first evidence of the timing of TH synthesis and release by fish thyroid tissue.

The interpretation of the physiological meaning of these phases is based on the assumption that the IS is, in fact, related to synthesis and secretory events. To test this hypothesis, we examined the pattern of IS in the thyroid tissue of two species, medaka (*Oryzias latipes*) [sexually immature adults] and rainbow trout [juvenile fish weighing approximately 12 g], following treatment of the fish with bovine TSH. For these studies we used two methods, the Vectastain horseradish-peroxidase method employing light microscopy, and an immunofluorescent method using confocal microscopy. In case of the rainbow trout, the fish were fed with T_4 or T_3 for several days prior to challenging with TSH in order to lower endogenous TSH production and thereby reduce or eliminate the secretion or release of endogenous TH. In the case of the medaka, the fish were sampled in March when the thyroid tissue is at a relatively low activity level. In both species, a progressive pattern of IS was found with an increase in staining response in the peripheral zones of the follicle lumen, particularly in the region of colloid vesicles, and then the appearance of IS in the cytoplasm of some thyrocytes (Raine, 1997; J.C. Raine, A. Takemura and J.F. Leatherland, unpublished data). These observations suggest that the temporal changes in the pattern of IS in the embryos are related to changes in the activity of thyroid tissue function, and lend support to the hypothesis that Phase 1 represents a period of thyroglobulin synthesis, associated with oxidative iodination and condensation, and Phase 2 involves the proteolysis of thyroglobulin to release the covalently-bound hormone.

For rainbow trout embryos, the onset of Phase 1 activity was coincident with the appearance of immunostainable (with anti-hTSH) pituitary thyrotrophs.

Moreover, between Phases 1 and 2 there was a marked proliferation in the number of thyrotrophs (Raine and Leatherland, 2000). This suggests that the initiation of TH synthesis in the trout is linked to the initiation of activity of the HPT axis. Thus, biotic and abiotic factors that influence the maturation of the HPT axis could potentially influence the timing of first synthesis and secretion of the embryo's own TH.

Effect of exogenous and maternal sources of TH on early development of the embryo

i. Exogenous hormone

The effects of immersion of teleostean embryos and juvenile stages in solutions of TH are well reported (literature reviewed by Reddy and Lam, 1992; Leatherland, 1994; Brown and Kim, 1995). Such studies result in an increased rate of development of early embryos, including a more rapid absorption of the yolk sac. Some reports also note that these TH-enhanced rates of development reduce the viability of the embryos and early juvenile stages, and deformity and mortality rates are high. Although these studies are clearly toxicological or pharmacological in nature, they do provide two important pieces of information. They show that the TH's can influence developmental events in fish embryos, both in terms of the rate of development, and the outcome of the developmental processes, and that the TR's are present very early in development, in most species before the appearance of the embryo's own thyroid tissue. Recent work dealing with the expression of TR genes in fishes supports this finding (see below).

The biological significance of this early presence of the TR and the responsiveness of embryonic tissues to TH's remains illusive. However, it may have marked significance in consideration of the actions of environmental toxicants, particularly the environmental endocrine disruptors (reviewed by Leatherland, 1998).

ii. Yolk hormone

Since the first reports of the presence of THs in the yolk of teleostean fish embryos (Kobuke et al., 1987; Tagawa and Hirano, 1987), there have been several studies of the changing patterns of yolk thyroid hormone content in a broad range of teleostean fish species (reviewed by Leatherland, 1994). In general, the pattern of change suggested a progressive decrease in 'whole body' TH content. However, although it was assumed that the TH measurements reflected the content of the whole organism (tissue, body fluids and thyroid), the patterns of change were not what would be expected if this were true. There was, for example, no evidence of an elevation of whole body TH content associated with the appearance of colloid in the thyroid tissue. Also, for two species studied in our laboratory (Arctic charr, *Salvelinus alpinus*, and rainbow trout), and for coho (*Oncorhynchus kistuch*) and chinook salmon (*Oncorhynchus tshawytscha*) (Greenblatt et al., 1989), sporadically high 'whole body' TH values were found in individual fish. In an attempt to explain that apparent paradox, we postulated that the 'whole body' TH measurements might not be measuring the covalently bound TH in the thyroglobulin and tested the hypothesis by digesting homogenates of the pharyngeal tissue with trypsin with the purpose of releasing covalently bound TH from thyroglobulin, thus making the molecules available for ligation with antibodies in T_4 and T_3 RIAs. After 1 hour digestion, there was a 100 fold increase in assayable T_4, and a 2-3 fold increase in assayable T_3, providing evidence in support of our argument that the extraction processes used had not, *ipso facto*, extracted the TH contained within the thyroglobulin. Thus, the 'whole body' TH measurements made in embryos and early juvenile stages of fishes to this time probably do not accurately reflect the TH contained within the thyroid tissue compartment.

In light of the effects of TH-administration on fish embryo development (see above), and the early appearance of TRs in fish embryos, the importance of the yolk hormones on the development of the embryo prior to the onset of thyroidogenesis by the embryo has been of considerable interest. Surprisingly, several studies failed to demonstrate an effect of altered yolk TH content on embryonic events (reviewed by Leatherland, 1994), suggesting that the yolk hormones may not be involved. However, it is possible that the yolk hormones exposure is regulated by the embryo by altering the rates of TH metabolized and excretion, and thus experimental variation in the yolk TH content may not change the levels of exposure of the embryonic tissues to TH. It is likely that the differences in responses of embryos to TH challenge by immersion in a solution of hormone and by altering levels of TH in the yolk are related to

ability of the embryo to control the circulating TH levels. Following immersion, TH will enter the blood via the gills, and the levels may exceed the ability of the embryos to metabolize and excrete excess hormone. In contrast, the transfer of hormone from the yolk into the somatic tissues of the embryos will likely be at a rate well within the ability of the embryo to maintain TH homeostasis.

Timing of the appearance of TRs and potential sites of action of THs

As discussed above, indirect evidence based on the responsiveness of embryonic tissues to TH challenge suggests an early appearance of the TRs in fish, probably before the thyroid tissue of the embryo has commenced thyrogenesis. Recent studies of TR gene expression in fishes and in *Xenopus laevis* support this evidence (Banker et al., 1991; Essner et al., 1997; Yamano and Miwa, 1998; Llewellyn et al., 1999), and, as discussed above, it is possible that the yolk THs, with the levels of exposure to the TRs regulated by TH metabolism and excretion, play a role in early developmental events. In addition to the biological importance of this process related to the control of early development, and the environmental toxicological relevance are considerable. Factors that influence the delivery of THs to developing tissues (by impacting the transport of hormones in the blood or across membranes) or compromise the binding of T_3 to the TR proteins can potentially disrupt embryonic development at key windows of opportunity and bring about irreversible changes (Porterfield, 1994).

Relatively little is known about the specific roles of thyroid hormones during early vertebrate development. Studies of the effects of hypothyroidism on early development suggest a marked influence of the THs in neurological development of neonatal mammals (reviewed by Porterfield and Hendrich, 1993; Cao et al., 1994), and recent studies of TR knockout mice have revealed new information of TH actions in mammalian embryos (reviewed by Hsu and Brent, 1998). Mice with TR gene inactivation exhibit very different responses compared with mice that have TR gene inactivation. The knockout mice show sensory and neurological impairment, growth arrest, delayed small intestinal maturation, cardiac dysfunction and thermoregulatory problems. Although the central and pleiotropic role of the THs in metamorphosis of amphibian tadpoles is well established, at this time, there is little specific information related to TH functions in fishes. With the increased understanding of the polymorphic nature of TRs, and an appreciation of the fact that not all actions of the THs are effected via genomic receptor sites (e.g., Davis and Davis, 1996), it is

anticipated that the cellular roles of THs in developmental processes will now begin to be defined.

Conclusion

The currently available evidence suggests that the THs can influence early developmental processes in teleostean fishes, and that TR protein is expressed in fish embryos in advance of the appearance of thyroid tissue in the embryo and considerably in advance of the onset of thyroidogenesis. In salmonid fishes the thyroid primordium is a tubular structure that does not take the form of a follicular glandular tissue until relatively late in embryonic development, and even in juvenile fish, the tubular primordium may still be present. Therefore, the use of criteria such as follicle counts as a means of assessing thyroid activity in early developmental stages of some species may not be appropriate. The onset of TH synthesis and release in salmonid fishes, as determined using immunohistological methods, occurs concurrently with the proliferation of pituitary thyrotrophs, and begins before the appearance of thyroid follicles. Relatively little is known about the cellular and tissue actions of the THs during early development of fish embryos.

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THE DEVELOPMENT OF ION REGULATION

IN THE RAINBOW TROUT

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These studies (the MSc thesis projects of K. Barrett and C. Misiaszek) examined the development of iono-regulatory mechanisms in rainbow trout from the eyed-egg stage (~28 days before hatching at 7° C) to the end of yolk sac absorption (~36 days after hatching) and up to ~25 days past the point of first feeding. Measurements of ion uptake (Na^+, Cl^- and Ca^{2+}) from the external medium, changes in tissue ion concentrations, and in specific ion activities (NH_4^+, H^+, Na^+ and Ca^{2+}) at the surfaces of the gills, skin and yolk sac were used to localize and characterize the ion transport mechanisms.

Na^+ regulation develops early relative to Ca^{2+} or Cl^- regulation. A saturable Na^+ transporter is clearly present by the eyed-egg stage and is responsible thereafter for the net accretion of Na^+ over the remaining period of embryonic development and throughout larval development. The transport mechanism continues to mature well into larval development with increasing affinity and capacity. In contrast, evidence for the net accretion of Cl^- and Ca^{2+} is ambiguous until the beginning of larval development. There appears to be only minimal changes to the Cl^- transport mechanism through larval development and the Ca^{2+} transporter shows a <u>decrease</u> in affinity with development. Both the chorion of the embryo and the surface of the larvae have a high binding capacity for Ca^{2+} which complicates the interpretation of findings regarding the Ca^{2+} transport mechanism.

Sampling the near surface environment of the larval trout with a Na^+ specific electrode showed that the gills are, from early in larval development, the main site of Na^+ loss, and therefore, by inference, the main site of Na^+ uptake. Moreover, Na^+ permeability increases in parallel with increases in Na^+ uptake through development, indicating a much larger self-exchange for Na^+ than for other ions. In contrast, Ca^{2+} loss appears to be non-specifically distributed to all larval surfaces. However, the apparent Ca^{2+} loss is more likely evidence of pronounced surface binding of Ca^{2+}. The presence of mitochrondrial rich cells (MRCs) in the yolk sac that show no evidence of Na^+/K^+ ATPase activity suggests that these cells might be specialized for Ca^{2+} transport while MRCs found in the gills are specialized for Na^+ (and Cl^-?) transport. Measurements of boundary layer acidification suggest that the whole body surface appears to contribute to CO_2 excretion with the gills playing a minor role. In contrast, ammonia excretion appears to be confined to the gills. However, we found that acute elevation of external Na^+ did not stimulate ammonia excretion, a finding that argues against the presence of a Na^+/NH_4^+ antiport in the gills.

The loss of the yolk sac and the onset of feeding prompts a further suite of changes in the iono-regulatory machinery particularly with respect to Ca^{2+}; a dramatic change in the surface binding properties for Ca^{2+}, a down-regulation of trans-epithelial Ca^{2+} transport and an increasing reliance on dietary Ca^{2+} to meet the demands of skeletal mineralization. This finding goes against the conventional wisdom that teleosts meet their Ca^{2+} requirements primarily through absorption across the gills.

We conclude that the regulatory mechanisms for each ion are qualitatively and quantitatively quite distinct from one another and may well be distributed to different MRC sub-types. The early development of the Na^+ transport mechanism and the fact that Na^+ uptake substantially exceeds that required for Na^+ balance suggests a prominent role for Na^+ in acid-base regulation (i.e. Na^+/H^+ exchange). The early development of an acid excretion mechanism may be necessary to clear acidic metabolites accumulated through embryonic and early larval development.

IONOREGULATORY DEVELOPMENT IN EARLY LIFE STAGES OF RAINBOW TROUT AND THE EFFECT OF SILVER.

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EXTENDED ABSTRACT ONLY-DO NOT CITE

Introduction

Active Na^+ uptake from fresh water has been demonstrated to occur in salmonid eggs starting at the eyed stage (Rudy and Potts, 1969) presumably driven by an apical H^+-ATPase in series with a basolateral Na^+,K^+-ATPase, as is thought to be the case in the fish gill (Lin and Randall, 1995). The first objective of this study was to investigate the pattern of ion regulatory development from fertilization to pre-swim-up, based upon immunolocalization of the Na^+,K^+-ATPase and V-type H^+-ATPase in the gills, skin and yolk sac epithelium of rainbow trout coupled with measurements of whole organism unidirectional Na^+ uptake and Na^+,K^+ATPase activity levels.

Silver acts as a Na^+ analogue and exerts its toxic effect by impairing branchial Na^+,K^+-ATPase in juvenile and adult fish, leading to loss of ion regulatory control and death (Wood et al., 1996). Very little is known of the effects of silver exposure on ion regulation in developing fish embryos and larvae which

are often the most sensitive to toxicants. Thus, the second objective of this study was to investigate the effects of chronic silver exposure on ion regulatory development in rainbow trout.

Materials and Methods

Freshly fertilized rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from Rainbow Springs trout farm (Thamesford, Ontario) and maintained in darkened chambers in flowing, dechlorinated Hamilton tap water at a constant temperature of 12 °C. Within 3 h following fertilization, up to 1 week post-hatch, eggs were continuously exposed to sublethal levels of silver (as $AgNO_3$) at 0, 0.1 and 1.0 μg/l total silver in a flow-through set up. Every 5 days, unidirectional Na^+ influx was measured (using ^{22}Na) and eggs or larvae were collected for measurement of whole organism Na^+, Cl^-, Ag^+, cortisol and ammonia levels as well as Na^+,K^+ ATP-ase activity determinations. In a separate series, eggs and larvae that had not been exposed to silver were collected every 5 days, fixed in Bouins fixative and sectioned for immunolocalization of the Na^+,K^+-ATPase and V-type H^+-ATPase in the gills, skin and yolk sac epithelium. Na^+,K^+-ATPase and V-type H^+-ATPase were indirectly immunolocalized using the mouse monoclonal α 5 antibody and the rabbit polyclonal anti-peptide (A-subunit) antibody, respectively (Wilson et al. 2000).

Results and Discussion

The ontogeny of ion regulation was investigated in rainbow trout from fertilization to swim-up, in the presence and absence of sublethal levels of silver, a Na^+ antagonist. Whole egg unidirectional Na^+ uptake increased dramatically from the "eyed stage" (10-15 nmol g h^{-1}) through to post-hatch and swim-up (350-400 nmol g h^{-1}) and this correlated well with the increase in whole egg Na^+,K^+ ATPase activity levels. Na^+,K^+-ATPase and V-type H^+-ATPase were immunohistochemically localized in the gills of pre-swim-up larvae not exposed to silver. While some labeling was also observed in the skin and yolk sac it was far less frequent indicating that the gills likely play the predominant role in driving active ion uptake at this stage of development. During exposure to sublethal levels of silver (0.1 and 1.0 μg/l total silver in hard water as $AgNO_3$) from fertilization to post-hatch, there is a dose dependent acceleration in growth and ionoregulatory development as indicated by changes in unidirectional Na^+ uptake and Na^+,K^+ ATPase activity levels (expressed per mg protein or per egg). Shortly following hatch, however, these sublethal levels of silver resulted in an

impairment of Na^+,K^+ ATPase activity at which time no significant differences in Na^+ uptake were observed among treatments.

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WHY FISH DEVELOP GILLS

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EXTENDED ABSTRACT ONLY -- DO NOT CITE

Introduction

It is generally assumed that fish develop gills to satisfy their respiratory demand for oxygen (Krogh, 1941). The reasoning goes that, because of surface-to-volume considerations, the metabolic demand for oxygen of embryos tends to increase at a faster rate than the surface area of the skin where the bulk of gas exchange takes place. The solution to this supply-demand problem is to develop gills where, because of their morphology, the same surface-to-volume considerations do not apply. The problem with this hypothesis is that many species develop gills long before they are large enough for cutaneous surface area to be the limiting factor in gas exchange (Rombough & Moroz, 1997). This raises the possibility that gills initially form for other reasons; the most likely of which is ionoregulation. This seems to be the case in rainbow trout (*Oncorhynchus mykiss*) where morphological indicators (surface area for gas exchange and chloride cell numbers for ionoregulation) strongly suggest that ionoregulation is the dominant function of the gill during larval development (Rombough, 1999). Morphological indicators, however, do not always accurately reflect physiological capacities so before we reject the oxygen hypothesis and 100 years of history it would seem prudent to obtain more direct evidence of early gill function. The goal of this study was to obtain such evidence.

Methods

The method used to determine which aspect of gill function, respiration or ionoregulation, is the *raison d'être* for gill formation was a variation on the

classic ablation study. Zebrafish (*Danio rerio*) larvae were effectively prevented from ventilating their gills either by exposing them to anaesthetics (tricaine methanesulphonate or phenoxyethanol) or by embedding their gills in agar. The larvae then were placed in media designed to selectively compensate for impaired oxygen uptake (42% O_2), impaired ionoregulation (50% physiological saline) or impairment of both functions (42% O_2 + 50% saline). Survival in these media was compared with survival in normoxic (21% O_2) fresh water.

Discussion

It was hypothesized that:

(1) if gills were required for oxygen uptake, hyperoxia would improve survival,
(2) if gills were required for ionoregulation, saline would improve survival,
(3) if both functions were required, survival would be best in hyperoxic saline.

Neither hyperoxia nor saline had any significant effect in newly hatch larvae (3 days postfertilization) suggesting that at this stage gills were not necessary for either oxygen uptake or ionoregulation. By the time the larvae are 7-d old, the gills appear to be required for ionoregulation but not gas exchange. Physiological saline significantly enhanced the survival of 7-day postfertilization larvae under both normoxic and hyperoxic conditions. At this stage hyperoxia still had no protective effect, either alone or in conjunction with physiological saline. The situation was similar for 14-d old larvae (i.e. saline but not hyperoxia extended survival). It was not until the larvae were about 21-d old that hyperoxia significantly extended survival times.

The fact that gills do not appear to be required for gas exchange until long after they are required for ionoregulation has broad implications. In particular, we may have to reassess how we view structure-function relationships in adult fish. It could also provide new insight into the evolutionary origins of the vertebrates.

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EFFECTS OF TEMPERATURE, SALINITY AND BODY SIZE

ON THE ROUTINE METABOLISM

OF SPOTTED SEATROUT (*Cynoscion nebulosus*) LARVAE

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Abstract

Routine oxygen consumption rates of larval spotted seatrout (*Cynoscion nebulosus*) were measured over a range of temperatures (24, 28, 30 and 32°C) and salinities (5, 10, 20, 35 and 45‰). Larvae (5.0-49.0 mm TL) varying over several orders of magnitude in dry body mass were used to estimate an allometric scaling relationship, resulting in a bi-phasic pattern in the mass scaling of metabolic rate. Oxygen consumption (μL O_2 larva^{-1} hr^{-1}) scaled isometrically with body mass (slope=0.997) for larvae <6.8 mm TL and allometrically (slope=0.797) thereafter. The inflection in the mass-metabolism relationship coincided with the formation of the hypural plate and a change in swimming mode. Temperature and salinity effects on routine oxygen consumption were analyzed using ANCOVA with larval dry weight as a covariate. Temperature and salinity significantly affected routine metabolism

during the second phase of growth only. A significant interaction between temperature and salinity was evident at 30 and 32°C during the second phase of growth. A response surface describing the environmental influences on routine metabolism was developed to provide a bioenergetic basis for modeling environmental constraints on the growth of this species. Ontogenetic changes in the mass scaling of metabolism are discussed in relation to the change in hydrodynamics experienced by larvae.

Introduction

The routine metabolic rate (M) can be expressed by the allometric equation $M=aW^b$, where a and b are constants. If b=1, then weight-specific metabolic rate remains constant, and total metabolism increases in proportion to weight. When b is less than 1, total metabolism increases more slowly than weight, and the metabolic rate per unit body mass decreases with increasing body size (Winberg, 1956). This decline in metabolic rate with size has been attributed to an increase in the relative mass of tissue with low metabolic activity (white muscle and bone), combined with a decrease in overall metabolic activity of tissues (Oikawa et al., 1991). If metabolic rate is proportional to body surface area, and different sized bodies are geometrically similar, the value for b should approximate 0.67 (Schmidt-Nielson, 1984). This theoretical value is rarely observed, but values closer to 0.75 are more common (Peters, 1983). Although the surface area is not important for thermoregulation in fishes, it is important for some fish larvae that utilize cutaneous respiration during the early life stages.

Ontogenetic changes in the relationship between body mass and metabolic rate have been reported by several investigators (Oikawa et al., 1991; Kamler, 1992; Post and Lee, 1996). It appears that in many fish species, the metabolic rates of early life history stages scale isometrically with body mass (Giguere et al., 1988). Post and Lee (1996) described a general bi-phasic pattern for the scaling of metabolic rate with body mass in several species of fish. They proposed metabolic rate increases directly proportional to body mass (isometric) early in life, and later increases less than proportional to body mass (allometric). The location, and importance of the inflection in this relationship, however, is unclear in most species. One possible explanation for the change in scaling of metabolism is the change in swimming efficiency during early development. The physical environment of fish larvae changes from a viscous flow regime to an inertial flow regime as they develop. A change in swimming style from anguilliform to subcarangiform or carangiform accompanies this change in hydrodynamic surroundings (Hunter, 1972). This change is likely to occur very

early in development, perhaps about the time of first feeding, and would not explain the prolonged isometric scaling of metabolism for some species, namely, salmonids. Marine species with small pelagic eggs and larvae will be more influenced by their hydrodynamic surroundings early in life. Spotted seatrout larvae hatch at a length of 1.65 mm TL and begin feeding at 2.6 mm TL (Alshuth and Gilmore, 1995), therefore viscous forces are likely to be important for these small stages.

Other physical properties of water affecting larvae are the temperature and salinity of the water. Spotted seatrout spend their entire life history within estuaries, and larvae are considered both eurythermal and euryhaline. Powell et al. (1989) collected larvae in salinities of 5-40 ‰ in and near Florida Bay, however, little is known on the metabolic cost incurred at different salinites. The presence of cutaneous chloride cells in early stage marine larvae enables them to maintain ion balance. Hiroi et al. (1998) demonstrated a shift in the distribution of chloride cells in Japanese flounder (*Paralichthys olivaceus*) from cutaneous to branchial chloride cells. This shift demonstrates the importance of cutaneous surfaces when larvae are small and in a viscous environment, before gills become an efficient means of ion exchange. We measured routine oxygen consumption to determine the relative metabolic costs for larval spotted seatrout over a range of temperatures, salinities and sizes. Specifically, we tested the following null hypotheses: 1) larval body mass has no effect on metabolism, 2) temperature has no effect on metabolism, and 3) salinity has no effect on metabolism.

Methods

Spotted seatrout larvae were obtained from the Texas Parks and Wildlife GCCA/CPL Marine Development Center in Corpus Christi, Texas. The captive broodstock were spawned using photoperiod and temperature cycling. After hatching, yolk-sac larvae were shipped overnight to the NOAA Laboratory in Beaufort, NC. Larvae were reared in circular 100 L tanks. When larvae developed pigmented eyes and a functional mouth, rotifers, *Brachionus* sp., were introduced at a concentration of 5 ml^{-1} along with algae, *Nannochloropsis* sp. or *Isochrysis* sp.

Routine metabolic rate was determined for 773 individuals at four temperatures, and five salinities. All fish were acclimated for 2-3 days prior to measurement of oxygen consumption. Artificial lights regulated to natural photoperiod were used throughout the experiment. In order to minimize the effects of diurnal

variation in metabolic rate, all oxygen consumption measurements were determined from 0800 to 1600 hrs. Experimental fish were removed (by pipetting) from culture tanks in the morning, before feeding, placed in filtered seawater of the appropriate salinity and temperature, and held for 1-2 hrs. to clear their gut. Trials were conducted in a Gilson differential respirometer on individual larvae (5.0-49.0 mm TL, 15-13190 µg dry weight) in 15 ml respiration flasks, following the procedures of Hoss et al. (1974). Larvae were allowed to acclimate for one hour in the flask before oxygen consumption measurements were made at regular intervals (0.5-1 hr) for a period of 2-6 hr depending on fish size. After each trial, fish were euthanized in MS-222, the notochord or standard length measured under a dissecting microscope, dried at 60°C, and the final dry weight determined.

The hydrodynamic environment experienced by larvae depends on their length and velocity, and the viscosity and density of water. The ratio of these inertial and frictional forces is summarized as the nondimensional Reynolds number (*Re)*:

$$Re = U*L/v$$

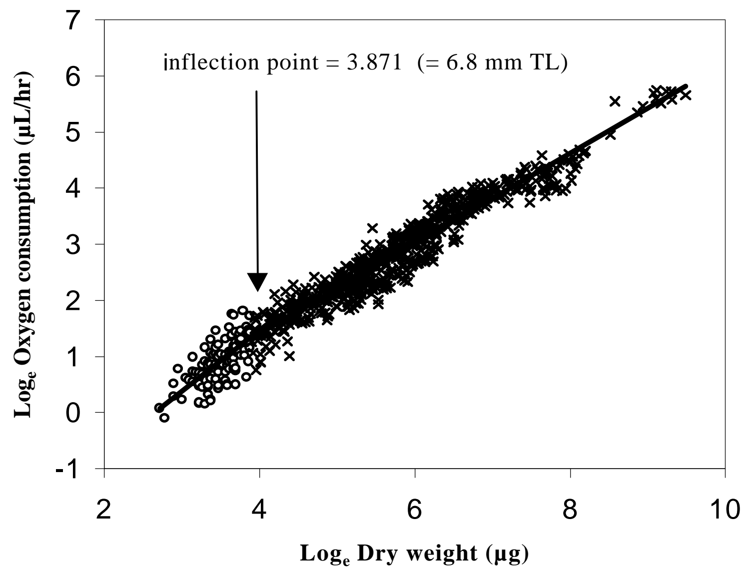
Where U is the velocity in body lengths (BL) sec^{-1}, L is the total length, and v is the kinematic viscosity (ratio of viscosity to density). When Re is less than 30 viscous forces dominate, and at $Re>200$ inertial forces dominate. An intermediate zone is recognized at $30<Re<200$, where the balance between the two forces gradually shifts from a viscous to inertial regime (Fuiman and Webb, 1988). Temperature and salinity both affect the Reynolds number, therefore these were held constant at 30°C and 20‰, and values of kinematic viscosity were taken from Pilson (1998).

The Log_e normalized metabolism data were subjected to a nonlinear, segmented fitting algorithm (SYSTAT, Wilkinson, 1990) to estimate the inflection point and two linear segments that best fit the data (Post and Lee, 1996). Temperature and salinity effects on the routine metabolic rate were tested in a factorial ANCOVA design with dry weight as a covariate, at a significance level of alpha=0.05.

Results

A bi-phasic relationship in the scaling of routine metabolism with body weight was evident, with the inflection occurring at 6.8 mm TL (Figure 1). The dry weight of the larvae explained most of the variance in oxygen consumption for

both phases of growth. During the first phase of growth, the routine metabolic rate scaled isometrically (slope=0.997). The metabolic rate scaled allometrically



(slope=0.797) for larvae greater than 6.8 mm TL.

Figure 1**.** Weight dependence of routine metabolic rate for larval spotted seatrout.

Reynolds numbers were calculated for seatrout through the larval period to relate the influence of swimming speed and body length on the hydrodynamic regime. It is evident that larvae do not escape viscous forces until they reach a length of 6-13 mm TL (Figure 2).

Neither temperature, salinity, nor the interaction term were significant (alpha=0.05) during the first phase of growth, possibly due to increased variability for smaller larvae. During the second phase of growth the effect of temperature was significant (P=0.0001). The interaction between temperature and salinity was also significant (P=0.0218) for larger larvae. A response surface (Figure 3) was generated to describe the oxygen consumption for a 299 µg spotted seatrout larvae in response to temperature and salinity.

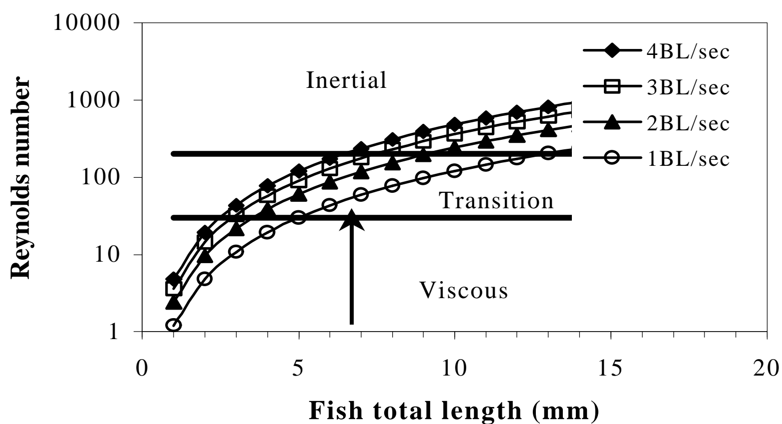


Figure 2. Reynolds numbers for spotted seatrout at different swimming speeds (temperature = 30°C, salinity = 20‰). Arrow indicates size at metabolic inflection point.

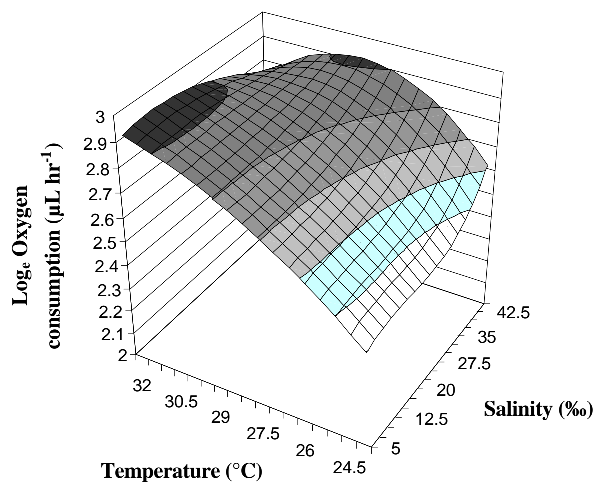


Figure 3. Response surface generated for spotted seatrout larvae during the second phase of growth (dry weight = 299 µg).

Discussion

A bi-phasic pattern in the mass scaling of metabolic rate was observed. The oxygen consumption rate (µL O_2 larva^{-1} hr^{-1}) scaled isometrically with body mass (slope=0.997) for larvae up until a size of 6.8 mm TL, and then scaled allometrically (slope=0.797) thereafter. The isometric scaling during the first phase of growth is consistent with the conclusions of Giguere et al. (1988) for the larvae of other fish species. The oxygen consumption rates over a wide range of larval size in this study enabled the evaluation of changes in the metabolic scaling. Based on data from this study, spotted seatrout follow the general model of metabolic ontogeny proposed by Post and Lee (1996). The inflection point for spotted seatrout is close to the value they report for sea bream, *Pagrus major*. For both of these marine fishes, the inflection is much lower than their estimates for two freshwater species, rainbow trout, *Oncorhynchus mykiss*, and common carp, *Cyprinus carpio*.

Notochord flexion occurs at 5.7 mm TL in spotted seatrout, and at 6.4 mm TL the urostyle bends upward and formation of the hypural plate begins (Alshuth and Gilmore 1995). The change in metabolic scaling at 6.8 mm TL coincides with the formation of the hypural plate. If we consider an average swimming speed of 2-3 BL sec^{-1} for larval fish (Blaxter, 1969), Figure 2 shows the correlation between the inflection point for routine metabolism and the change in hydrodynamic regime experienced by the larvae. These changes in the structure and surroundings of larvae enable them to change swimming modes from anguilliform, to the more efficient subcarangiform or carangiform modes, where increased weight becomes advantageous. This offers them an increased efficiency with size, and may account for the decreasing weight-specific metabolic rate (slope <1) with size after the inflection.

The effect of temperature on the routine metabolism was found to be significant during the second phase of growth. The effects of salinity was temperature dependent, evident by the significant interaction between temperature and salinity at 30 and 32°C during the second phase of growth. The theoretical cost of osmoregulation over a range of salinites calculated by Eddy (1982) was less than 1% for salmonids. Therefore, the energetic cost of ion regulation is likely to be very low, provided functional chloride cells are present. The overall response to salinity is probably affected by other metabolic processes. The response surface describing the influence of environmental factors on the routine metabolism provides a bioenergetic basis for modeling the environmental

constraints on the spatial and temporal growth of this species. The decrease in metabolic rate at high temperatures and salinities does not necessarily indicate increased growth under these conditions. Stressors which reduce metabolic rate may be accompanied by a decrease in consumption, therefore, growth would not be enhanced (Rice, 1990). The effect of high temperatures and salinities on the growth of spotted seatrout larvae cannot be determined until the consumption under these conditions is better understood.

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THE EFFECT OF THE GASEOUS ENVIRONMENT

ON THECARDIOVASCULAR DEVELOPMENT

OF TILAPIA, *Oreochromus niloticus*

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

We are interested in the developmental plasticity of the cardiovascular system. Cardiovascular development has drawn a lot of attention recently, probably because the heart is one of the first organs to function and the cardiovascular system one of the first organ systems to operate in any vertebrate embryo. Even so, few data exist for heart rate during development in fish. Even fewer data exist for the development of stroke volume and cardiac output, the latter being the most important variable for oxygen transport. A few studies have looked at the effects of altering the environment of developing fish embryos and larva, although most of these focus on the effects of acute changes to the environment, rather than morphological or physiological plasticity induced by prolonged exposure.

Methods

The purpose of this experiment was to test whether convective blood flow is necessary at early life stages for the delivery of oxygen to the tissues, and whether the development of the cardiovascular system is sensitive to the environment through feedback from O_2 delivery. In order to explore these

questions, tilapia (*Oreochromus niloticus*) larvae were raised in one of four environments: normoxia, 8% O_2, 2% CO, and a combination of 8%O_2 and 2%CO.

Tilapia eggs were obtained from the tilapia hatchery at Dalhousie University, Halifax, N.S., immediately after fertilization. Each spawning was separated into four groups and placed in one of four 10 gallon closed system treatment tanks: normoxia (control); 8% O_2 (hypoxia); 2% CO (which acts to functionally ablate Hb); and a combination of 8% O_2 and 2% CO (combination). Starting at 120 hours post fertilization, the larval hearts were video taped though an inverted microscope, and heart rate (hr = beats/min, bpm), stroke volume (sv = µl), and cardiac output determined (co = hr*sv = µl/min). The experiment was terminated when heart was no longer visible through the pericardial wall. Dry mass of the individual larvae was also determined (mg).

Results

Larval growth rate was affected by respiratory environment right from the start of the experiment, with the control fish growing faster than the other three treatments, CO and hypoxic fish being the same size and falling in the middle of the 4 treatments, and combined CO and hypoxic fish being the smallest of the treatments. This is probably due to the fact that tissue hypoxia decreased metabolism, and thus anabolic pathways, and the effect increased with increased limitation.

Heart rate of the tilapia larvae was unaffected by neither age nor treatment, however the effects of altering the respiratory environment on stroke volume and cardiac output (figure 1) are seen right from the start of the sampling. The data indicate that the cardiovascular system is required for delivery of oxygen to tissue at early stages of development in tilapia, and this need is dependant on stage and environment. Hemoglobin appears to be more necessary at later life stages than earlier ones and this is probably due increased importance of the cardiovascular system in delivering oxygen to tissues. Hypoxia, however, has more of an effect at the earlier stages. The effect of the combined CO and hypoxic environment was greater than hypoxia alone, indicating that cardiovascular oxygen transport seems to be important in tilapia at all life stages in hypoxic environments.

Discussion

The data obtained in this experiment on the effect of the respiratory environment on cardiac output suggest that cardiac output in larval fish is regulated by local tissue-blood vessel interactions acting through Starling effects of venous return. The absence of the bradycardia seen in adult fish in response to hypoxia implies that there is little neural input to the cardiovascular system in these larval fish. The greatly increased stroke volume is probably due to an enlargement of the heart. Growth regulation of the heart could be metabolic or mechanical. For example, hypoxia can dilate vascular beds, resulting in increased blood volume returning to the heart, and an increased stroke volume due to the Starling mechanism (Wadsworth 1994). Increased blood flow and hypoxia inducible factor 1 (HIF1) have been suggested to affect cardiovascular growth and remodeling during normal development, through factors such as vascular endothelial growth factor (VEGF) (Wenger and Gassmann 1997, Ratcliffe et al. 1998).

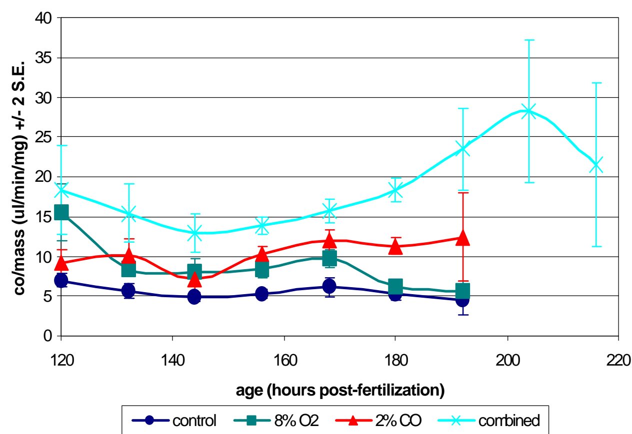

Figure 1. Mass specific cardiac output (co/mass) over time post fertilization for tilapia larvae exposed to four separate gaseous environments during development: normoxia (control); 8% O_2; 2% CO; a combination of 8% O_2 and 2% CO (combined). Standard error bars that do not overlap indicate significant differences between points.

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IS CARDIOVASCULAR OXYGEN TRANSPORT NECESSARY

IN FISH EMBRYOS AND LARVAE?

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Embryonic fish and amphibians have a functional heart and circulation, and often circulating red cells, well before hatching. Nonetheless, recent experiments suggest that the cardiovascular system has little role in oxygen transport in early developmental stages. For example, the distribution of oxygen uptake does not parallel the distribution of cutaneous blood vessels in Atlantic salmon yolk-sac larvae (Wells and Pinder, 1996). Moreover, carbon monoxide (CO), which prevents oxygen binding to hemoglobin , does not affect the distribution of oxygen uptake between gills and body surface, indicating that oxygen is not transported from the gills to other tissues (Pinder, Wells, and Sethi, unpublished data). Carbon monoxide also does not affect development, metabolic rate, or heart function in zebrafish embryos and larvae (Pelster and Burggren, 1996). Amphibian larvae have been raised to metamorphosis in CO, with little effect on growth, survival, metabolic rate, or cardiovascular development (Territo and Burggren, 1998). Cardiovascular oxygen transport is thus not necessary for normal development in several fish and amphibian embryos and larvae.

All of these examples are of fish and amphibians in normoxic water, which provides a relatively large PO2 gradient for direct diffusion to tissues, and a high enough PO2 to dissolve a significant amount of O2 in plasma. There are circumstances, however, that seem likely to require hemoglobin and cardiovascular oxygen transport: large body size, environmental hypoxia, and high temperatures (thus high metabolic rates). Indeed, skate embryos, which can reach several grams, have specialized external gills (there is no point in

having gills without cardiovascular oxygen transport from gills to tissues), as do embryos of viviparous perch (Webb and Brett, 1972). Embryos of some tropical frogs have external gills that are resorbed within minutes of hatching into tadpoles and whose resorption is slowed by hypoxia (Warkentin, pers. comm.). Foster (this symposium) demonstrates that carbon monoxide does affect growth and cardiovascular development in Tilapia, a fish with large embryos, developing at 29ºC. In brown trout at 15º (close to their upper temperature tolerance), treatment with CO decreases oxygen conductance by around 50%, indicating that roughly half the oxygen consumed is transported on hemoglobin (Pinder, Wells and Sethi, uunpublished data).

There are thus circumstances in which oxygen transport in fish embryos and larvae can be entirely by direct diffusion to tissues, and others where cardiovascular transport of oxygen bound to hemoglobin is important. The relative importance of these two parallel routes of oxygen transport will depend on several factors, primarily metabolic rate, embryo size, and environmental PO2. Circumstances in which cardiovascular oxygen transport is most likely to be important include all ovoviviparous or viviparous fish, fish with large eggs, fish reproducing in stagnant shallow water, and tropical fish. Circumstances least likely to require cardiovascular oxygen transport include marine pelagic eggs and larvae, temperate and arctic fish, and eggs and larvae developing in flowing water. These factors have long been speculated to affect the degree of cardiovascular development seen in different species of fish. We may now be able to test these ideas by combining detailed studies of individual larvae with accumulated data on egg sizes and environmental conditions for large numbers of species. Although the first tests for an oxygen transport function of the cardiovascular system in fish larvae suggest that the cardiovascular system is less important than earlier assumed, there are still very few studies addressing the question, representing only a tiny proportion of the diversity of fish development.

Acknowledgements

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TO LARVAE AND BEYOND:

GROWTH AND METABOLISM IN DEVELOPING FISHES

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Growth is energetically costly. For fish embryos and larvae which have growth rates that often exceed 20-30% per day, costs of growth are expected to be high. In the relationship between mass (M) and metabolic rate (VO_2), (i.e. VO_2=aM^b), where a is the mass coefficient and b is the mass exponent, b usually ranges between 0.69-0.80 and mass scales allometrically with metabolic rate. Conversely, in the relationship $VO_2/M = aM^b$ where VO_2/M is the metabolic intensity or mass-specific metabolic rate, metabolic intensity decreases with increasing mass or with growth and b is negative and approximately – 0.2. In contrast with these well-known physiological relationships recent studies on fish embryos and larvae found that metabolic rates scaled isometrically with mass, (i.e. mass exponents ranged from b = 1 to b> 1), and that metabolic intensities were independent of mass and absolute growth rate (Giguere et al. 1988, Weiser and Medgyesy 1990a, Rombough 1994, Weiser 1994). The majority of these studies used freshwater species which have large eggs, well developed larvae with low mortality rates which develop rapidly to juveniles. Much fewer studies have used marine species that have much smaller eggs, less developed larvae high mortality rates (>90%) and develop slowly to juveniles. For this reason few studies has investigated the effect of development and growth on metabolic-mass relationships across all early life history stages in a marine species.

The present study investigated the metabolic-mass relationships from embryonic to juvenile stages in Atlantic cod (*Gadus morhua*) and tested the premise that metabolic – mass relationships scale isometrically in all three early life history stages.

Methods and Materials

For eggs and larvae, measurements of oxygen consumption were made using a four-channel closed-system semi-flow-through respirometric system equipped with pulsed polygraphic oxygen electrodes (Type 1125, Endeco Inc., MA) housed in 250 µl Plexiglas chambers. After each respirometry experiment eggs and larvae were removed from the chambers and dried (n= 20-30 eggs, n=10 larvae, for each mass estimate). For juveniles respirometric systems consisted of three 2.8l transparent round plastic containers with equipped with a pulsed polygraphic oxygen electrodes. For all respirometric studies, metabolic rates and metabolic intensities were calculated from regressions of oxygen concentration over time for each electrode. For each larval respirometric run swimming activity was recorded using a video camera and images were analyzed using a digital analysis program to provide estimates of swimming speeds (Hunt von Herbing and Boutilier 1996). For juveniles, fish were allowed to swim freely in the chambers and rates were compared with speeds that approximated maximal activity rates (White 2000). Estimates of swimming activity provided data, which suggested that both larvae and juveniles were swimming at routine rates.

Results and Discussion.

Metabolic rate, like tissue mass, increased rapidly in an exponential pattern during the embryonic and larval periods. Metabolic rates continued to increase throughout development and after metamorphosis to the juvenile stage and mean metabolic rates were 2 fold greater in juveniles (e.g. 0.200 ± 0.0056 $mgO_2\ h^{-1}$) than in larvae and embryos. Metabolic expansion (i.e. the rate at which metabolic rate increases with mass) appeared to vary as a function of stage. For embryos, larvae and juveniles metabolic expansion appeared to be to be allometric. In embryos the metabolic mass exponent was b = 0.67, but the relationship was weak (p=0.046) (SE= 0.306; r^2_{adj}=0.22, n=15). For larvae b= 0.84 (SE= 0.65, r^2_{adj}=0.53, n=65) and for juveniles b= 0.86 (SE= 0.17; r^2_{adj} = 0.54, n = 21) and b was significantly less than unity (p<0.0001).

Comparisons of metabolic mass exponents (b) among all stages, showed that significant differences in slope existed (ANCOVA, p<0.0001). Embryonic mass exponents were significantly different from both larval and juvenile exponents. However, metabolic mass exponents between larvae and juveniles were not significantly different. For metabolic intensity, when all three stages were analyzed separately, only embryos showed a significant negative relationship

($p<0.05$) between metabolic intensity and mass ($r^2= 0.602$, SE = 0.636). For larvae and juveniles, relationships between these two variables were not significantly different ($p>0.05$) from zero. However when stages were combined, relationships between metabolic intensity and mass were significant ($p<0.001$) for the combined embryonic-larval-juvenile periods (b= - 0.27, SE=0.02) and for the combined larval-juvenile periods (b = - 0.29, SE=0.018) and exponents were very close to those predicted by general physiological relationships (i.e. - 0.2).

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EFFECT OF SEA WATER DESALINATION WASTE PRODUCTS ON FISH DEVELOPMENT

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

The aim of this study was to measure the effect of desalination waste brine on the early life stages of turbot S*cophthalmus maximus* L. Water desalination is very important in many countries where fresh water is scarce. Many desalination plants have been built and are functioning in many parts of the world. These plants separate salt from seawater using several techniques. The end product is fresh water that goes to the people and hypersaline hot brine that is poured back to the sea. This contains several substances like corrosion products, antiscaling additives (polycarbonic acids, polyphosphates), antifouling additives (chlorine and hypochlorite), halogenated organic compounds formed after chlorine addition, antifoaming additives, anticorrosion additives, oxygen scavengers (sodium sulfite) causing oxygen deficiency, acid, heat and the concentrate which is the excess salts. (Hopner *et al*. 1996). Del Beve (1994) states that extremely high salinities (twice the ocean ambient) can impact ocean biota if they are exposed for extended time periods. For brine discharged near the benthos, biota could be exposed to high salinities for as long as the effluent continues. To elucidate the effect of brine discharged from a desalination plant upon coastal marine organisms, (Iso *et al*.,1994) carried out preliminary experiments on the incipient lethal high salinity (ILHS) on some stages of development such as fertilised eggs, larvae, juveniles and adults, of two species of fish and a bivalve.

They found that the ILHS was about 50‰ or even higher (ranging 50-70‰) and that the high salinities caused delays in embryonic development.

Materials and Methods

All the fertilised and non fertilised turbot eggs were purchased from Manin Farms from Isle of Man

Preparation of solutions:
All the solutions were made from normal filtered seawater.

Boron Solution with 50‰ salinity:
The boron solution was made by dissolving Di-sodium tetraborate in filtered seawater. Artificial salt was added to the solution until it reached the wanted salinity which was 50‰. The solution was then aerated for 2 or 3 hours before being used each time.

Preparing 50‰ salinity solution:
This was prepared by adding artificial salt until arriving at the required salinity.

50‰ and pH6 solution:
The 50‰ solution was prepared, as above, then 10% HCl was added drop by drop until pH6 was reached. The acidity was measured by a pH meter. Then the salinity was measured again. The acidity and salinity of this solution was measured each time before use.

pH6 solution
10% HCl was added to normal sea water drop by drop until the required acidity ph6 was reached, and it was measured by a pH meter.

Streptomycin sulphate and penicillin were added to all the solutions (1mg/L). All solutions were well aerated each time before use.

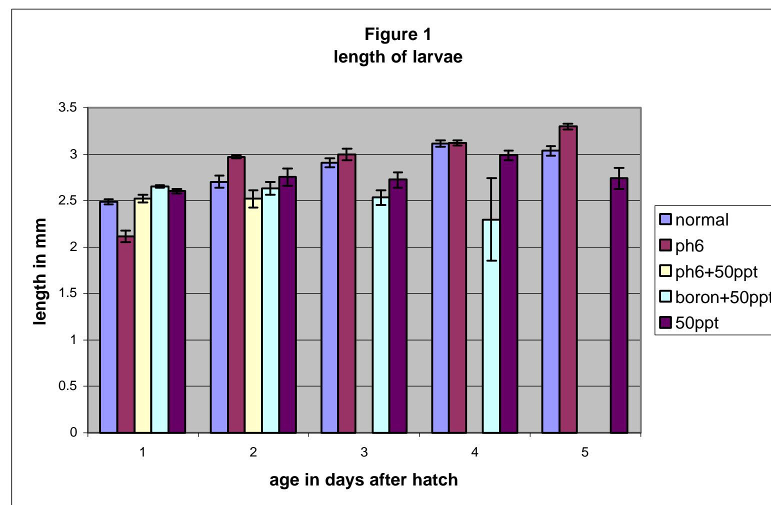
Each day specimens were taken from each tank using a Pasteur pipette. The samples were fixed into Bouin's fixative and 10% formalin in seawater, in order to make whole mounts and histological sections from these samples. Some samples were frozen with their solutions for physiological analysis.
Every day live specimens were video taped to record the actual shape and activity. A stereo microscope connected to a video camera was used.

Histological wax sections were made and stained with Haematoxylin & Eosin.The sections were then photographed for measurements eg. Body wall and eye cup measurements which were done using an image analysis program (Image tool).

Results and discussion:

High salinity solutions caused high death rates in embryos and larvae. Larval length was affected by high salinity but not by acidity (Fig 1).

It was also clear that the larvae in hypersaline solutions used up their yolk sac content more quickly than controls.



The body wall was thicker in the larvae reared in hypersaline solutions compared with the controls.

The larvae treated in pH6 hatched prematurely, and had much fewer pigment cells in the body wall compared to the controls.

The larvae, which were treated with the boron+50‰ solution, had a C shaped body, showing some malformation in the vertebral column (Fig 2).

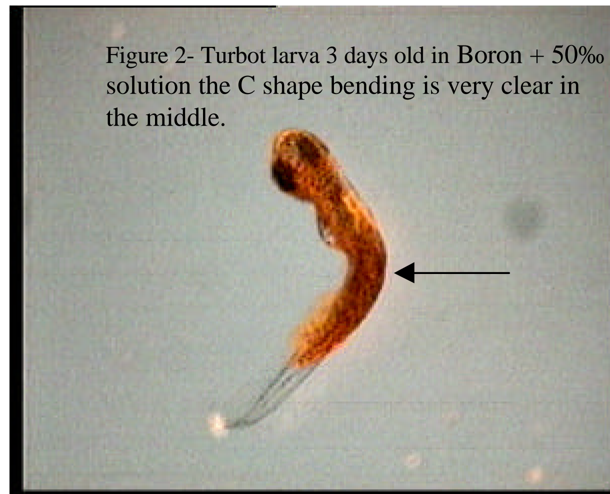


Figure 2- Turbot larva 3 days old in Boron + 50‰ solution the C shape bending is very clear in the middle.

We conclude from this work, that the desalination waste brine has direct teratogenic effects on turbot embryos and larvae.

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