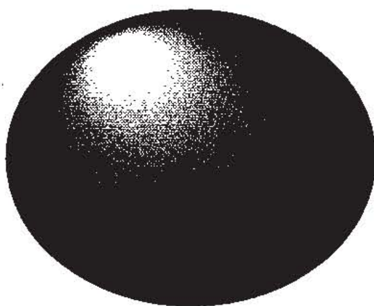
The Fish Egg:

Its Biology and Culture

Symposium Proceedings

Don MacKinlay

Maxwell Eldridge



International Congress on the Biology of Fishes

San Francisco State University July 14-18, 1996

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PREFACE

In the disciplines of fish biology and fisheries biology, few other subjects have experienced the growth and interest as has the area of early life stage biology — namely, fish eggs and larvae. Its rapid rise in popularity can be seen in comparing the size, content and list of references of J.H.S. Blaxter's chapter on fish eggs and larval development in the 1969 Fish Physiology Volume III with the specificity and diversity of an entire volume (same series, Volume XI) devoted to the subject, published in 1988. Today, one need only peruse any of the major "fish" journals and there will be a significant number of articles focusing on eggs and/or larvae. Ichthyoplankton surveys and studies of larval fish ecology have become essential to fisheries and natural resource conservation and management. Egg and larval development and physiology are integral parts of studies in aquatic ecology. Early life stages of fish now serve as standard experimental models for many research fields, including pollutant effects, toxicology, pharmacology and developmental biology. It is no wonder that a call for papers for an egg symposium should elicit such a positive response.

Yet our original preconception for this symposium, as part of the International Congress on the Biology of Fishes, was to have a series of papers that would cover "egg" topics that would deal with issues, problems and research relating mostly to aquaculture (read 'hatcheries') and to salmonids. Much of the explanation for this position was due to the public and governmental concern, and thus funding, over the declining stocks of Pacific coast salmon. To our pleasant surprise, the collection of papers submitted for presentation soon made us aware that our preconception was a misconception. The international scope of interest extended far beyond our regional perspective and, in fact, reflected a full diversity of disciplines.

The approximate 32 papers comprising this symposium come from 13 countries, representing four continents. Topics are international and diverse, covering a full phylogenetic range of species from freshwater, estuarine and marine habitats. Under the general heading of fish egg, there are papers on pre-fertilized oocytes and the traditional early life stages of embryos and larvae. In an attempt to logically organize the various papers, they appeared to fall into three general categories: effects, egg quality, and development and physiology.

Under the topic of effects, we examine how extrinsic factors such as temperature, pH, petrochemicals, mechanical shock, pathogens and transport handling affect fish eggs and larvae. The broader category of egg quality explores more intrinsic factors that include the determinants of egg size, and varied nutritional components such as lipids, proteins, and free amino acids. In the last section on egg development and physiology, topics range from traditional descriptions of development to functional studies on factors that influence egg and larval development, metabolism and physiology.

The success of this symposium was the result of the combined effort of all the contributors and their shared concern for and scientific interest in some of the most interesting critters around — fish eggs and larvae. Without them, we would not have adult fish to enjoy.

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CONGRESS ACKNOWLEDGEMENTS

This Symposium is part of the International Congress on the Biology of Fishes, whose main sponsors were Fisheries and Oceans Canada (DFO), the US National Biological Service (NBS) and San Francisco State University (SFSU). The main organizers of the Congress, on behalf of the Physiology Section of the American Fisheries Society, were Alec Maule of NBS (overall chair and registrations), Don MacKinlay of DFO (program and proceedings) and Ralph Larson of SFSU (local arrangements). I would like to extend a sincere 'thank you' to the many contributors who took the time to prepare a written submission for these proceedings. Your efforts are very much appreciated.

Don MacKinlay

TABLE OF CONTENTS

Effects on Eggs and Larvae

The effects of egg size and incubation temperature on the hatching and early growth of larval herring. *Morley, SA and RS Batty* 9

Thermal marking of alevins to enable identification of hatchery stocks. *MacKinlay, DD, CC Cross, K Dover and W Hoyseth* 19

The effect of temperature on the rowth and yolk conversion efficiency of Atlantic salmon alevins from three European rivers. *McCarthy, I, D Houlihan and L Hackney* 25

Sensitivity to mechanical shock in Atlantic salmon eggs during their first six hours after fertilization. *Krise, WF* 35

Early life-stage outbreaks of systemic bacterial cold-water disease — is the causal agent *Flexibacter psychrophilus* vertically transmitted in salmonids? *Brown, LL, W Cox and RP Levine* 39

Some teratogenic and pathologic impact on fish and embryos in Lake Mariut. *Zaki, MI, MI Michael and SG Ghabrial* 45

Viability of milkfish eggs and larvae after simulated and actual transport. *Toledo, JD, M Doi and MN Duray* 51

Developing spawning and incubation techniques for migratory mahseer in the Himalayan rivers of Nepal. *Shrestha, TJ* 59

Egg Quality

Parental size and perceived brood value: are all eggs created equal? *Galvani, AP and RM Coleman* 67

Evolution of egg size in neotropical cichlid fishes. *Coleman, RM* 73

Free amino acids and protein content in pelagic and demersal eggs of tropical marine fishes. *Ronnestad, I, R Robertson and HJ Fyhn* 81

Synthesis of free and conjugated 17α, 20β-dihydroxy-4-pregnen-3-one by embryos of Arctic charr. *Khan, MN, R Renaud and JF Leatherland* 85

Lipid and protein changes during egg and embryo development in the viviparous genus *Sebastes*: application to the assessment of reproductive success. *MacFarlane, RB and EC Norton* 95

Egg quality of grouper fed different acid sources. *Quinitio, GF, RM Coloso, NB Caberoy, D Toledo and DM Reyes* 103

Egg size determines offspring size in neotropical cichlid fishes. *Coleman, RM and AP Galvani* 109

Development and Physiology

Delayed hatching in the terrestrial eggs of grunion. *Martin, KLM, RA Darkin and MC Fisher* 121

Oogenesis in *Sparus aurata*. *Ramos, MA* 125

Development and energy utilization in early life stages of viviparous yellowtail rockfish.
Eldridge, MB and BM Jarvis 131

Embryological characteristics of two *Oreochromis* spp. and their hybrid using the induced spawning method. *Zaki, MI, MI Michael and SG Ghabrial* 141

Direct evidence of physotomous gas bladder inflation in physoclistous fish larvae.
Rieger, PW 149

Embryonic and larval development of *Puntius schwanefeldii*.
Harmin, SA and J Jais 157

Temperature and efficiency of development during endogenous feeding in herring embryos and yolk sac larvae. *Overnell, J* 161

A specialized role for the Pacific herring egg chorion in sperm motility initiation.
Vines, CA, et al. 167

Study of milt of Puye.
Valdebenito, I, et al. 173

In vitro cortisol metabolism by embryonic tissues of Arctic charr and rainbow trout.
Khan, MN, PK Reddy, R Renaud and JF Leatherland 179

Effect of hCG dosages on hatching success in white bass.
Kohler, CC, RJ Sheehan, A Suresh, L Allyn and J Rudacille 189

Effects on Eggs and Larvae

THE EFFECTS OF EGG SIZE AND INCUBATION TEMPERATURE ON THE HATCHING AND EARLY GROWTH OF LARVAL HERRING

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Introduction

Egg size variation has been well studied in N.E. Atlantic herring, *Clupea harengus*, stocks (Almatar and Bailey, 1989, Hempel and Blaxter, 1967 and Blaxter and Hempel, 1963). Egg size not only varies between herring stocks which spawn in different seasons and at different geographical locations but also amongst individuals within the same spawning stock. This variation in reproductive strategy between investing more yolk in fewer eggs and investing less yolk in more eggs will affect the characteristics of hatching larvae and therefore their probability of survival. This study investigated various hatching characteristics of three N.E. Atlantic herring stocks.

Several studies have shown that longer larvae hatch from larger eggs (Marteinsdottir and Able, 1992, mumichog; Blaxter and Hempel, 1963, herring). Swimming speed is positively correlated to larval length (Batty and Blaxter, 1992) and therefore a larvas ability to feed and escape from predators will be affected by initial egg size. Larvae have also been shown to hatch with larger yolk reserves and more body tissue from larger eggs (Beacham and Murray, 1985, Chum salmon; Blaxter and Hempel, 1963, herring). Increased body reserves would be expected to increase the time to starvation of a larva (Blaxter and Hempel, 1963) and give the larva a better chance to encounter a patch of food at suitable density before total yolk sac resorption. However, Chambers *et al* (1989) found no relationship between the quantity of yolk reserves of capelin and the expected post hatch survival time in the absence of food.

Temperature on herring spawning grounds varies considerably from year to year. For example a 42 year series of temperatures taken from the North Channel, an area close to Ballantrae Bank (the spawning site of the Clyde herring used in this study), shows that the mean temperature during March ranged from 4.8-9.8°C (Jones and Jeffs, 1991). The temperature that eggs and larvae experience will affect development by altering the rates of many biochemical and physiological processes (Blaxter, 1993).

Temperature can act directly to effect the survival of embryos even within their zone of tolerance, both higher survival at lower temperatures (Forrester and Alderdice, 1966, Pacific cod) and an optimal survival temperature (Beacham and Murray, 1985, Chum salmon) have been reported. Temperature also has a direct effect on time to hatch (Hempel and Blaxter, 1967). Egg size has been reported to both interact with temperature to affect hatch time (Pauly and Pullen, 1988) and have no

effect on the temperature hatch time relationship (Miranda et al, 1990, sardine; Beacham and Murray, 1985, chum salmon; Blaxter and Hempel, 1963, herring).

Incubation temperature is reported to have a range of effects on the hatch length of fish larvae. Embryos are either not affected by incubation temperatures (Blaxter, 1956, Clyde herring) longer at hatch when incubated at a higher temperature (Forrester and Alderdice, 1966, Pacific cod; Blaxter and Hempel ,1963, Clyde herring; Blaxter, 1956, Buchan herring), or longer at hatch when reared at a lower incubation temperature (Beacham and Murray, 1985, Chum salmon; Blaxter and Hempel, 1961, German coastal herring and Meyer, 1878, Baltic herring).

The above studies largely look separately at the effect of either temperature or egg size on the developing embryo. This study aims to investigate the effect of the interaction between egg size and temperature on: survival; time to hatch and length, weight and yolk volume on hatch.

Materials and Methods

Percentage survival

Ripe adult herring were caught off their spawning grounds and embryos incubated in the laboratory following the techniques of Blaxter (1968). 10 eggs were counted from each female, dry weights measured and egg batches covering a wide range of egg sizes were selected for each stock. Larvae were incubated at the temperatures shown in table 1.

Stock	Year	Temperature regimes/°C	Egg dry weights, mg
Buchan (Bu94)	Autumn 1994	8, 12 and 15	0.12-0.19
Manx (Mn94)	Autumn 1994	10 and 13.5,17	0.28-0.4
Clyde (Cl94)	Spring 1994	5, 8 and 12	0.28-0.39
Clyde (Cl95)	Spring 1995	5, 8 and 12	0.3-0.44

Table 1. Incubation details

Three replicate microscope slides of Buchan eggs for each of 32 females and at each temperature were photographed at intervals from fertilization until hatching. In each photograph the number off eggs surviving as a proportion of the number of eggs fertilized was counted.

Hatching characteristics

Before hatching eggs were transferred to 1 litre floating cylindrical containers with a 63µm mesh floor to facilitate water exchange. Larvae were removed daily, counted and a sub-sample of, where possible, 10 larvae from each egg batch were measured for the following parameters: total length; the maximum yolk sac width and length (from which yolk volume was calculated using equation 1); and dry weight.

Equation 1: $$\frac{4}{3}\pi * \frac{1}{2}(yolklength) * \frac{1}{2}(yolkwidth)^2$$

Daily measurements were continued until peak hatching and mean values on the day of peak hatch were used for further analysis.

Variable name	Variable type	P>F
1/temp.	continuous	<0.01
Stock	class	0.20
Egg size	continuous	0.70
Overall model		<0.01

Table 2. The results of a SAS GLM analysis of covariance model on the date of 50% hatch of larvae. No interaction terms were significant.

From the data of the number of larvae hatching on each day the date of 50% cumulative larval hatch was calculated and this was rounded up to the nearest day. Greater accuracy could not be used as larvae could only be counted daily as they hatch at night. The SAS procedure GLM (SAS Institute Inc., 1988) with an analysis of covariance model was used to test the significant factors affecting the hatch date distribution, and each of the three hatching characteristics, total length, dry weight and yolk volume.

Results

There was no clear relationship between egg dry weight and percentage survival of Bu94 eggs incubated at 15, 12 or 8°C. Survival of eggs reared at 8°C, 25.12±12.04 (±1S.D) was generally higher than that of fish reared at either 12°C, 6.71±7.40, or 15°C, 2.73±3.45.

The SAS GLM model showed that there were no effects of stock or egg size on the date of 50% hatch of larvae, Table 2. Time to hatch, for all stocks, was related to the inverse of incubation temperature, Equation 2.

Equation 2:

$$HatchTime = \frac{140.01}{incubationtemperature} - 0.71$$

Stock	Variable name	Length	Weight	Yolk volume
Bu94	Temp.	<0.01	<0.01	<0.01
	Egg size	<0.01	<0.01	<0.01
Mn94	Temp.	NS	NS	NS
	Egg size	<0.01	<0.01	<0.01
Cl94	Temp.	<0.01	NS	0.02
	Egg size	<0.01	<0.01	<0.01
Cl95	Temp.	<0.01	<0.01	0.05
	Egg size	<0.01	<0.01	<0.01

Table 3. Results of SAS GLM procedure for each stock separately. Temp. = incubation temperature (a class variable) and Egg size is a continuous variable. NS = non significant variable. All models were significant to P<0.01.

In all cases longer larvae hatched from larger eggs, Figure 3 and Table 3, and the length of larvae at peak hatch also depended upon the incubation temperature in all cases except Mn94, Figure 3b. For a given egg size, embryos incubated at the high temperature were shorter than larvae incubated at the medium temperature which in turn were shorter than larvae reared at the low temperature.

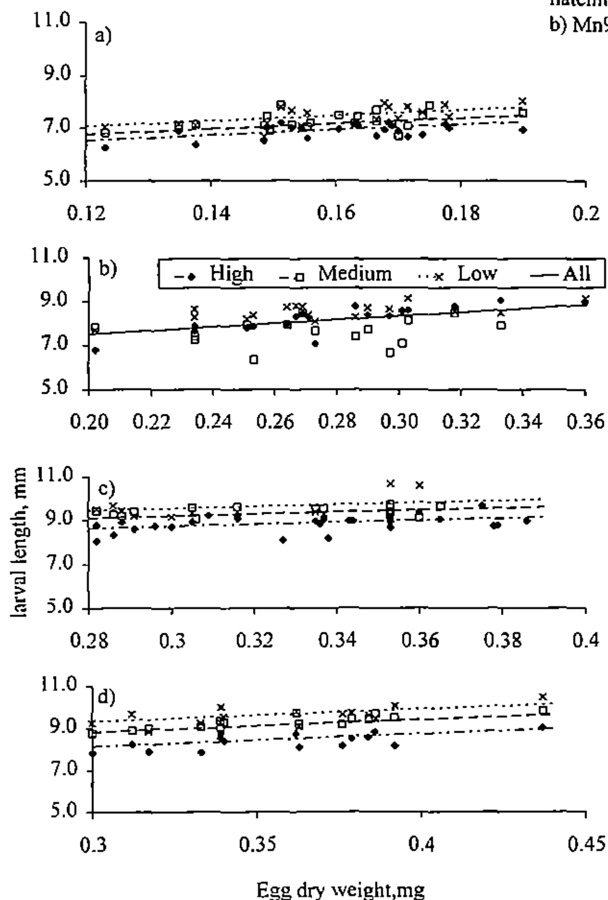

Figure 3. The effect of egg dry weight and rearing temperature on hatching length for a) Bu94 b) Mn94 c) Cl94 and d) Cl95.

Figure 4 and table 3 show that for all stocks heavier larvae hatched from larger eggs. Also, for a given egg size, Bu94, Figure 4a and Cl95, Figure 4d, larvae were heaviest at hatch when incubated at the low temperature whilst those incubated at the high temperature were the lightest.

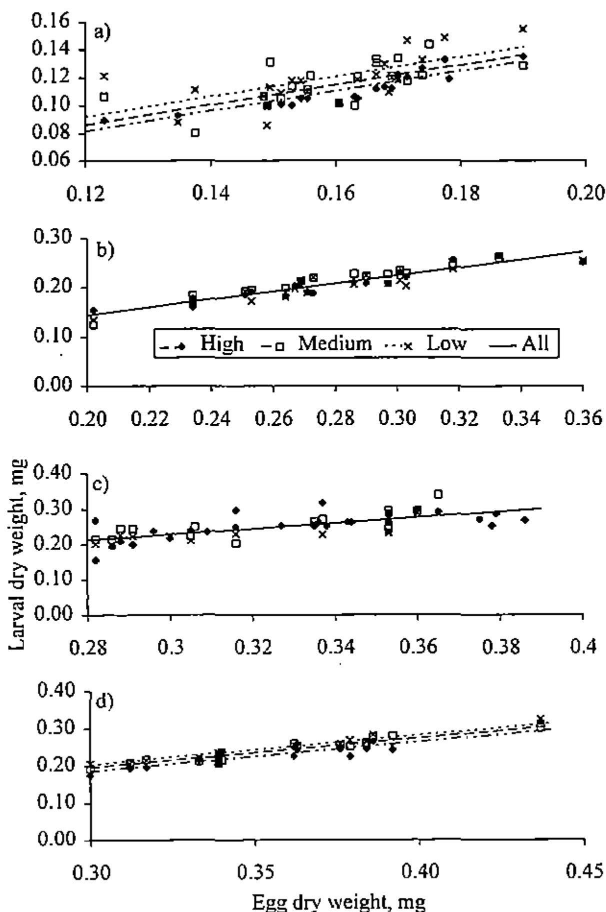

Figure 4. The effect of egg dry weight and incubation temperature on hatching weight for a) Bu94 b) Mn94 c) Cl94 and d) Cl95.

Figure 5 and table 3 show again that in all cases larvae hatched from larger eggs had more yolk. There are again small effects of incubation temperature on the amount of yolk larvae hatch with, but again not for Mn94 embryos. These effects are also reversed between Cl95 and the other two stocks. Bu94 and Cl94 embryos incubated at the low temperature hatched with the most yolk whilst low temperature Cl95 larvae hatched with the least yolk. The same but reverse effect was true for larvae incubated at the high temperature.

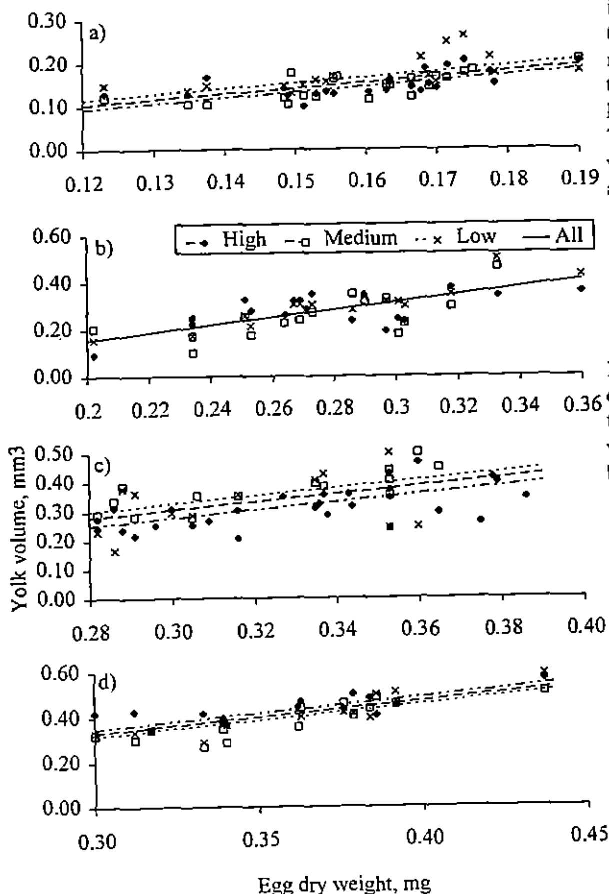

Figure 5. The effect of egg dry weight and incubation temperature on hatching yolk volume for a) Bu94 b) Mn94 c) Cl94 and d) Cl95.

Variable name	Variable type	Ln (length)	Ln (weight)	Ln(yolk volume)
Temp	Class	<0.01	0.20	0.33
Stock	Class	0.84	0.63	0.29
Ln(egg)	Continuos	<0.01	<0.01	<0.01

Table 4. Significance values for SAS GLM analysis of covariance models for the effects of rearing temperature (Temp) stock and egg size (Ln(egg)) on total length, weight and yolk volume.

Table 4 shows that SAS GLM models can be fitted to the ln(length), ln(weight) and ln(yolk volume) data where there is no effect of stock. Ln(egg size) has a

significant effect on all three characteristics whilst temperature only had an overall effect on length, Figure 6. The longest larvae hatched when incubated at the low temperature and the shortest larvae hatched from the high incubation temperature, Figure 6a. The fitted line for hatch length has a slope of 0.30 and therefore egg size has a greater effect on hatch length for the smaller eggs in this study. The fitted line to the egg size/hatching dry weight relationship has a slope of 1.00 so an increase in egg dry weight of 1 mg will result in an increase in larval weight of 1 mg, Figure 6b. The fitted line for yolk has a slope of 1.16. Larvae therefore hatch with an increasing proportion of the egg yolk in the form of yolk sac with increasing egg size.

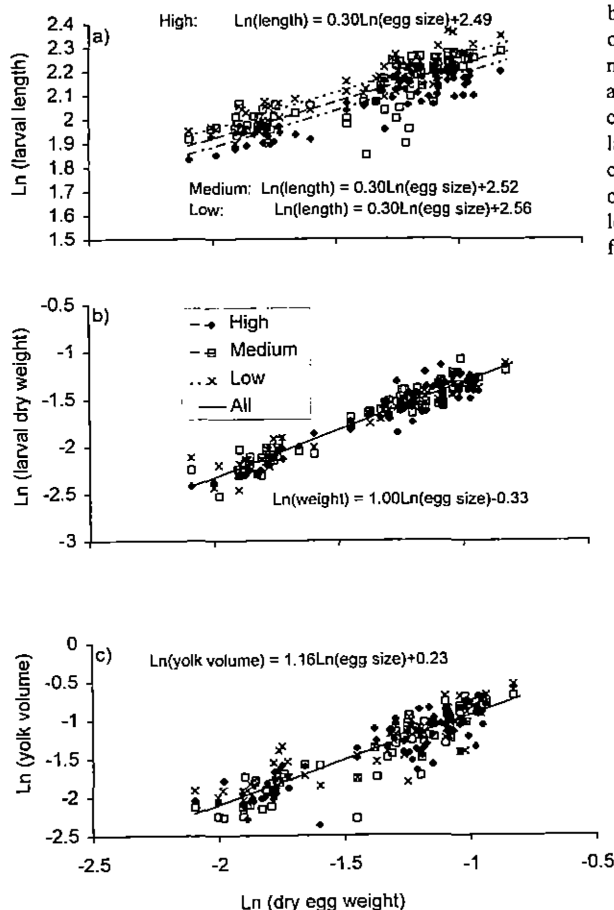

Figure 6. The relationship between natural logarithm of egg dry weight and the natural logarithms of a) length, b) weight and c) yolk volume, of hatching larvae from all stocks combined. Temperature only had an overall effect on length. SAS GLM model fits are shown.

Figure 7 shows that for Cl95 larvae which were reared on beyond hatching larvae reared at low temperature generally remain longer for a given weight whilst larvae reared at high temperature remain generally shorter for a given weight.

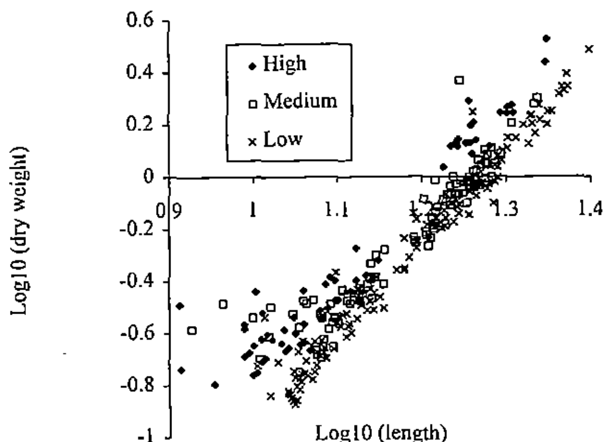

Figure 7. The length weight relationship of larvae incubated and then reared at three temperatures.

Discussion

Incubation temperature had a very similar effect on the timing of hatch to that found by Blaxter and Hempel (1963). Larvae incubated at 14.5°C hatched between 6 and 10 days post fertilization (Blaxter and Hempel recorded a value of 7.5 days at 14°C) larvae incubated at 5°C hatched between 23 and 25 days post fertilization (Blaxter and Hempel recorded a value of 24 days post fertilization).
In addition we have demonstrated that both egg size and spawning stock do not affect the hatching time nor was there any interaction between temperature and egg size affecting the hatching time.

Survival of Manx embryos was highest at the lowest incubation temperature (10°C). Although they suspected that all their treatments were affected by hypoxic conditions Forrester and Alderdice (1966) suggest that survival of pacific cod was higher at lower incubation temperatures because of the lower oxygen requirements of larvae developing more slowly at these temperatures. Frequent water exchange meant that hypoxic conditions were not a factor during our study. If eggs had been incubated at progressively lower incubation temperatures then survival would eventually decline when the temperature drops below the embryos zone of tolerance.

Munk and Rosenthal (1983) showed that egg density had affected the hatching characteristics of Baltic herring. They suggested that the contact area of each egg with the surrounding water will determine the oxygen available to that embryo and markedly affect the embryos physiology and subsequent hatching characteristics. In this experiment eggs were scattered randomly over glass plates to avoid large clumps. However, there could still be some differences in oxygen availabilities between embryos which might account for some of the variation in the relationship between egg size and hatch characteristics in this experiment.

There was a strong relationship between egg size and the three hatching characteristics, total length,

weight and yolk volume which could be described by one relationship for all stocks. Length has an asymptotic relationship with egg size which therefore has a steeper slope for smaller egg sizes. Therefore, small changes in egg size have more effect on hatching Buchan larvae than they do on Clyde larvae. This suggests a mechanism where by stocks laying small eggs would have larvae with a wider range of hatching characteristics perhaps making them more likely to produce at least some larvae capable of surviving in widely fluctuating environments. Blaxter and Hempel (1963) found that the Baltic herring stock which have the smallest eggs also have the highest variability in larval size at hatching. The fact that the increased weight of larger eggs is transferred directly into larval weight suggests that whilst in the egg embryos are able to convert the energy stored as yolk quite efficiently. However, this study also found that a greater proportion of the weight of these heavier larvae remains in the form of unutilised yolk still in the yolk sac. This could be an adaptation to give them longer to find food before yolk sac exhaustion in a poor environment.

Temperature variations will clearly interact with egg size to effect the hatching characteristics of larvae, particularly in the case of hatch length. The lack of an overall effect of temperature on weight and yolk volume on hatching suggests that temperatures, over the ranges tested in this experiment, have no effect on the efficiency of yolk conversion. However, the effect of temperature on weight and yolk volume of hatching larvae for combined stocks will be obscured, to a certain extent, by the differences between stocks. In particular the effect of temperature on yolk volume was reversed between years with the same stock. These inter-stock differences and a lack of an overall pattern are not surprising considering the temporal and spatial variation between the spawning stocks.

Larvae are not only longer at hatch for a given size of egg when incubated at lower temperatures but remain longer for a given body weight during the early growth phase at these same temperatures. This suggests itself as a possible mechanism where larvae subjected to lower temperatures remain longer for a given body weight and are therefore able to attain higher swimming speeds to compensate, to some extent, for their reduced swimming speeds at these temperatures.

References

Almatar, S.M. and Bailey, R.S., 1989. Variation in the fecundity and egg weight of herring (*Clupea harengus* L.) Part I studies in the Firth of Clyde and northern North Sea. .-J. du Conseil 45: 113-124

Batty, R.S. and Blaxter, J.H.S. 1992. The effect of temperature on the burst swimming performance of fish larvae. *J. exp. Biol.*, 170, 187-201.

Beacham, T.D.,and Murray, C.B. 1985. Effect of female size, egg size and water temperature on developmental biology of Chum salmon (*Oncorhynchus keta*) from the Nitinat River, British Columbia. *Can. J. Fish. Aquat. Sci.* 42: 1755-1765.

Blaxter, J.H.S. 1993. The effect of temperature on larval fish. *Neth. J. Zool.*, **42**: 336-357.

Blaxter, J.H.S. 1968. Rearing herring to metamorphosis and beyond. *J. mar. biol. Ass. U.K.* **48**: 17-28.

Blaxter, J.H.S. 1956. Herring rearing - II. The effect of temperature and other factors on development. *Mar. Res. Scot.* **5:** 19pp.

Blaxter, J.H.S. and Hempel, G. 1963. The influence of egg size on herring larvae (*Clupea harengus*). *J. Cons. Int. Expl. Mer.* 28: 210-240.

Blaxter, J.H.S. and Hempel, G. 1961. Biologische Beobachtungen bei der Aufzucht von Heringsbrut. *Helgoländ. Wiss. Meeresunters.* 7: 260-283.

Chambers, R.C., Leggett, W.C. and Brown, J.A. 1989. Egg size, maternal effects, and the correlations between early life history traits of capelin (*Mallotus villosus*) (Pisces: Osmeridae): An appraisal at the individual level. *Rapp. P.V. Reun. Ciem.* **191**:

Forrester, C.R. and Alderdice, D.F. 1966. Effects of salinity and temperature on embryonic development of the Pacific cod (*Gadus macrocephalus*). J. Fish. Res. Bd. Canada. **23(3)**: 319-340.

Hempel, G. and Blaxter, J.H.S. 1967. Egg weight in Atlantic herring. *J. Conseil* **31**: 170-195.

Jones, S.R., and Jeffs, T.M. 1991 Near-surface sea temperatures in coastal waters of the North Sea, English Channel and Irish Sea. *Fish. Res. Data Rep.*, Fish. Lab. Lowestoft, Suffolk, **24**: 1-70.

Marteinsdottir, G. and Able, K.W. 1992. Influence of egg size on embryos and larvae of *Fundulus heteroclitus* (L.) *J. Fish Biol.* **41**: 883-896.

Meyer, H.A. 1878. Beobactungen über das Wachsthum des Herrings im westlichen Theile der Ostsee. *Jber. Komm. Untersuch. Dtsch. Meere Kiel*, **4-6**: 229-250.

Miranda, A., Cal, R. M. and Iglesias, J. 199O. Effect of temperature on the development of eggs and larvae of sardine *Sardina pilchardus* Walbaum in captivity. *J. Exp. Mar. Biol. Ecol.* **14O**: 69-77.

Munk, P, and Rosenthal, R. 1983. Variability in size of herring larvae at hatching. Influence of egg deposition patterns of parental fish. I.C.E.S., C.M. 1983/L:33. pp 16.

Pauly, D.P. and Pullin, R.S.V. 1988. Hatching time in spherical, pelagic, marine fish eggs in response to temperature and egg size. *Env. Biol. Fishes.* **22(4)**: 261-271.

SAS Institute Inc. 1988 *SAS/STAT users' guide. Release 6.03 ed.* Cary, North Carolina.

THERMAL MARKING OF ALEVINS TO ENABLE IDENTIFICATION OF HATCHERY STOCKS

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Abstract: The contribution of hatchery releases to returning adult chinook salmon in the Chilliwack River has been assessed using two independent methods. Fish that swim in to the Chilliwack River Hatchery are counted and the count is adjusted by the proportion of marked versus unmarked fish that were released as smolts to estimate the enhanced contribution. The total number of fish that spawn in the river is estimated from dead pitch sampling expanded to account for only 8-12% of total return being accessible to the dead pitch, estimated from a previous Peterson tag-recapture study. The number of tagged fish in the dead pitch is expanded by the tagged-untagged ratio of the released smolts. These estimates do not account for differential survival between tagged, untagged and wild spawned fish, differential return locale preferences of wild versus hatchery origin returning adults and the potential errors in the total wild spawner return estimates. By adjusting incubation temperatures on an evenly timed schedule, we induced the formation of dark bands on the otoliths of alevins and were thus able to mark all of the hatchery-origin fish with no stress to the fish and at neglible cost. When these fish return in 3-5 years, we will be able to sample the wild and hatchery returns to obtain an independent and unbiased reading of the ratio between wild and hatchery-origin fish, and therefore gain a better understanding of the contribution that the hatchery makes to the spawning population.

Introduction

The Chilliwack River fall chinook run began as a transplant in 1981 of 675,000 eggs from the Harrison River run collected at the Chehalis River Hatchery. Transplants ceased in 1984, when

sufficient adults returned to the Chilliwack River Hatchery for fall run broodstock.

This run is assessed annually with a coded wire tag program, in which recoveries take place in the various commercial, sport and native Indian fisheries. The methods used are described by Kuhn et al (1988) and come under the auspices of the Mark Recovery Program (MRP), an international cooperative accounting of salmon production from hatcheries in the eastern Pacific. All coded-wire-tagged fish have their adipose fin removed so that marked fish can easily be identified as adults. The escapement to the river for natural spawning and to the hatchery for broodstock are also assessed.

Natural spawning escapement sampling consists of a dead pitch to estimate both total escapement and the proportion of fish of hatchery origin. The total escapement estimate is based on a comprehensive Peterson tag-recapture program (Ricker, 1981) conducted in 1986 which estimated that approximately 12% of the total returns to the river were accessible to the dead pitch sampling. Dead pitch recoveries are expanded by this factor to get the total estimated escapement. The hatchery-origin contribution originates from the expansion of coded wire tagged recoveries from the MRP. In some years, the enhanced hatchery-origin has accounted for 100% of the natural spawners, while in others it has ranged from 54 to 86% (Figure 1.).

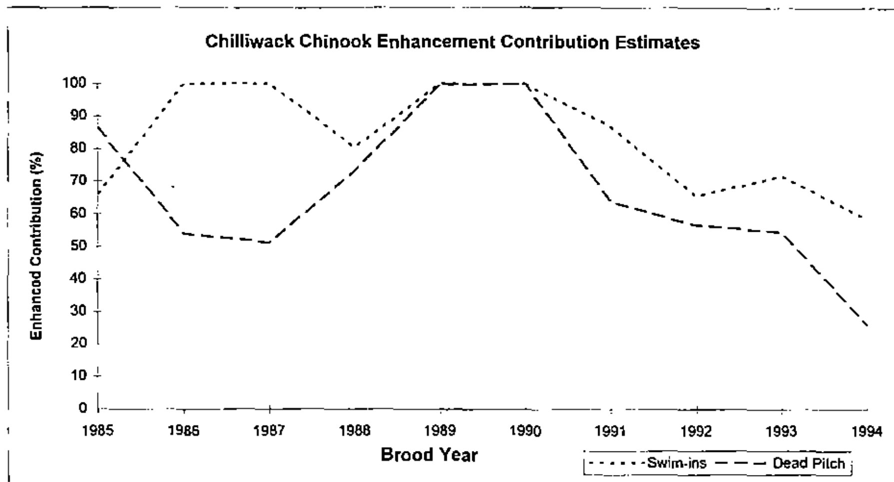

Figure 1. Results of different methods of estimating hatchery and wild-spawn contributions to Chilliwack River and Hatchery returns.

The hatchery-origin proportion of the fish that swim in to the hatchery is estimated by expanding the number of marked fish (no adipose fin) counted by the ratio of marked-to-unmarked fish released from the hatchery in the applicable brood years. All of the marked fish are eventually identified as to which release group they belong by reading the coded-wire tag.

Considering these data, it would appear that naturally spawning fish, although out-produced by the hatchery, are contributing to the escapement and that the transplant is now naturally propagating. However, difficult escapement sampling conditions have often rendered the estimated proportion

of hatchery origin questionable. In any event, there was often a large discrepency in the results from the two methods of estimating the hatchery and wild-spawn contributions.

In order to further examine the natural versus hatchery contributions, 1995 brood year hatchery fish were marked with a thermal otolith mark, as will be some subsequent years. Escapement will then be sampled, starting in 1998, for otolith marked fish so that hatchery and wild contribution can be independently estimated. Because every hatchery-origin fish will have an otolith mark, relatively small sub-samples of the wild and hatchery-return escapement should give statistically accurate estimates of their respective contributions to the total escapement.

Methods

The thermal marking procedure was based on the work of Brothers (1985) and Volk (1994) and refined into a stepwise procedure by Hoyseth (1995). The basic procedure is to decrease the incubation temperature by at least 2°C for 24 hr and then return to the original temperature for at least another 24 hr. A sequence of temperature drops and increases causes an increase in the amount of calcium laid down in bone growth rings during the lower temperature phase, resulting in alternating dark and light rings visible at the margin (which later are found near the center) of the otolith.

To get a consistent mark for a stock of fish, the thermal marks should be applied at approximately the same stage of egg/alevin development, so that the mark will be laid down in the same area of the otolith for each fish. Most of the chinook salmon eggs at the Chilliwack hatchery are taken over a period of a month, so the eggs were grouped into three different groups, approximately 10 days apart in their timing. The alevins were marked at 10 day intervals so that they would be close to the same stage of development during the marking procedure (Figure 2.).

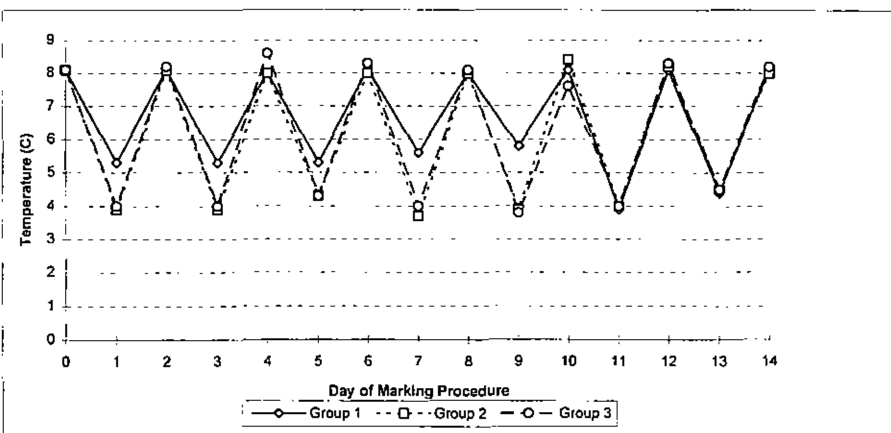

Figure 2. The temperature profile used to induce a thermal mark on chinook salmon alevins at the Chilliwack River Hatchery for the 1995 brood year. The chart shows the daily mean temperature for each day of the marking procedure. It actually took only one hour to adjust the temperatures, not a gradual adjustment as might be interpreted from the chart.

The temperature drops in the incubation water at the Chilliwack Hatchery were accomplished by switching from a mixture of well and ambient river water to only ambient river water, and then switching back to a mixture. The temperature pattern used to mark the alevins shows that at least the minimum drop of 2°C was attained with each change of water supply.

Samples of the fish were taken at the alevin and rearing (just prior to release at 6 g) stages to check that a valid mark had been imprinted on the otoliths. The fish were fixed in 90% denatured alcohol and shipped to the reading laboratory. Otoliths can be removed from fresh, frozen or preserved specimens. Preservation in 80-100% alcohol is considered very important because formalin seems to disintegrate otoliths. Sagittal otoliths were removed from the ear canals of the fish using a dissecting microscope, cleaned and affixed to a microscope slide with thermoplastic cement, melted on a hotplate. These small otoliths from alevins and juveniles were mounted sulcus side up, while otoliths from adults are mounted sulcus down. The otoliths were then wet-ground to mid-plane and polished using geological lapping film (a special type of fine sandpaper) of decreasing grit size (approx. 60μ to 1μ). Larger otoliths are ground with coarser paper (approx. 300 grit to 600 grit) on a machine to mid-plane, flipped over, and ground on the other side to mid-plane. They are then polished for examination.

The otoliths were examined under a compound microscope (100X to 400X) for the presence of thermal marks. Careful measurements were made using an eyepiece micrometer and photographs were taken of each otolith. Distances from the centre of the otolith (core) to the start of the basemark, basemark width, and focus to accessory mark were taken for each sample. The measurements were taken in the same quadrant of the otolith and abnormalities were noted. These procedures are meant to give a thorough profile of the thermal marks in the juveniles, so that they can be correlated to the marks found in returning adults, wherever they are captured.

Results

This paper only reports on the first phase of this experiment, that of placing and verifying the thermal marks on the alevins and juvenile fish before their release from the hatchery. A very good mark was obtained using the method described above. We had some equipment problems that did not allow us time to produce photographs of the 7-banded otoliths from this experiment, but we have included photographs of a 4-band otolith produced with the same method (Figure 3).

When the adult salmon return from the ocean, they will be sampled for otoliths by taking random samples from the populations swimming into the Chilliwack River Hatchery and those spawning in the Chilliwack River. Since all of the hatchery-origin fish will have a thermal mark, the escapement sampling program will only have to sample a small proportion of the returning fish to obtain a statistically valid estimate of the enhanced contribution. This will give an independent, and more accurate, estimate of the proportion of the fish that are hatchery or wild-spawner origin.

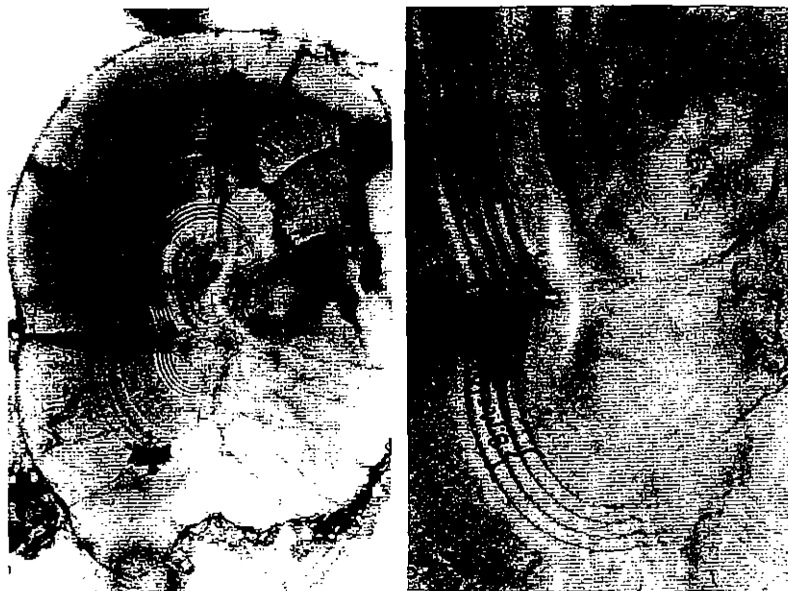

Figure 3. Photographs of a 4-band thermal mark from a chinook salmon at 200X (left) and 400X) right, showing the even distribution from daily temperature changes for four days.

Acknowledgements

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References

Brothers, EB. 1985. Otolith marking technique for the early life stages of lake trout. Great Lakes Fishery Commission, Research Completion Report, Ann Arbor MI.

Hoyseth, WJH. 1995. General procedures for thermal otolith mark induction and recovery. Pacific Biological Station. Fisheries and Oceans Canada. Nanaimo BC.

Kuhn, BR, L Lapi and JM Hamer. 1988. An introduction to the Canadian database on marked Pacific salmonids. Can. Tech. Rep. Fish. Aquat. Sci. 1649: vii + 56 p.

Ricker, WE. 1975. Computation and interpretation of biological statistics of fish populations. Can. Bull. Fish. Aquat. Sci. 181:xvii + 382 p.

Volk, EC, SL Schroder and JJ Grimm. 1994. Use of a bar code symbology to produce multiple thermally induced otolith marks. Trans. Amer. Fish. Soc. 123: 811-816.

THE·EFFECT OF TEMPERATURE ON GROWTH AND YOLK CONVERSION EFFICIENCY OF ATLANTIC SALMON ALEVINS FROM THREE EUROPEAN RIVERS

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ABSTRACT

The effects of water temperature on the developmental rate, nitrogen balance and yolk conversion efficiency of Atlantic salmon alevins from three different rivers stocks within the latitudinal range of the species in Europe (Iceland, Norway, France) was examined. The time taken from hatching to first feeding and size at first feeding decreased with increasing water temperature. Yolk-nitrogen conversion efficiencies decreased and standard metabolic rate at first feeding increased with increasing water temperature.

INTRODUCTION

Due to their poikilothermic nature, water temperature is the major abiotic factor regulating developmental processes in fish and the effects of water temperature on larval fish development have been the subject of several recent reviews (Blaxter, 1992; Rombough, 1996). In salmonid fish, elevated water temperature increases growth rate thereby reducing the alevin period and the relationship between temperature, aerobic metabolism, growth and yolk conversion efficiency has been studied for a number of Pacific salmon species (Rombough, 1988, 1994). In Atlantic salmon, *Salmo salar* L., the effects of water temperature on the time taken from hatching to first feeding and size at first feeding have been well studied (Petersen *et al.* 1977; Gunnes 1979; Brännäs 1988; Crisp 1988; Kane 1988; Jensen *et al.* 1989; Petersen & Martin-Robichaud 1995). However, there are very little data on the effect of temperature on yolk utilisation efficiency (Hayes & Pelluet 1945; Petersen & Martin-Robichaud 1995), aerobic metabolism or nitrogen budget during the alevin phase. The aim of this study was to examine the effect of water temperature on the developmental rate, nitrogen balance and yolk conversion efficiency of Atlantic salmon alevins from three different rivers stocks within the latitudinal range of the species in Europe (Iceland, Norway, France).

METHODS

Approximately 2000 eyed eggs, obtained from MSW Atlantic salmon (*Salmo salar* L.) from three European river stocks, the Alta in Finnmark in Northern Norway (70°N, 23°E), the Laxa in Adaldal in North East Iceland (66°N, 17°30'W) and the Allier in Southern France (45° 30'N, 3° 25'E) were imported into Aberdeen and reared at 2.5, 7.0 and 12.5°C until first feeding (Table 1). A random sample of 100 eggs from each river were sampled before the eggs were divided into three and the egg weight (to 0.1 mg), egg diameter (to 0.1 mm) and egg volume ($V=(4/3)\pi r^3$, mm^3) of these 100 eggs measured. The volume of yolk 24 hours after hatching was calculated by measuring the length and height of the yolk and using the formula for a prolate sphere, $V=(\pi/6)LH^2$. Alevins were sampled from each tank 24 hours after hatching and then at regular intervals between hatching and first feeding. On each sampling date ammonia excretion (mg NH_3/day) was measured for each river/temperature group (triplicate groups of 10 alevins) using a Phillips ammonia electrode. Each group was placed in 50 ml of freshwater at the appropriate water temperature and left for 2 hours. At first feeding, the standard metabolic rate (μM O_2/g/day) of fry was measured in 5 replicate groups of 5 fish from each river/temperature group using a Rank Oxygen Electrode connected to a Linseis pen recorder as outlined in Pannevis & Houlihan (1992). On each sampling date, 20 alevins were sampled at random and alevin weight (body + yolk, mg) and body total length (mm) were measured. The fish were placed individually in eppendorf vials, frozen in liquid nitrogen and stored at -20°C until analysis of the body and yolk protein content. Subsequently, the yolksac was carefully dissected away from the alevins whilst still frozen. The thin epithelial and syncytial cell layers that enclose the yolk ventrally were included with the yolk sample in the dissection of fish with an obvious yolksac. However, once the fish had 'buttoned up' it was possible to fully separate the yolk and body samples. At first feeding, a small amount of yolk remained visible within the peritoneum but it was not possible to separate this from the body and therefore the yolk was included in with these samples in any subsequent analysis. In order to comply with Scottish Office requirements for importation, waste water was treated with hypochlorite prior to disposal and the fish remaining at first feeding killed and incinerated together with all mortalities and waste material.

RESULTS AND DISCUSSION

a) DEVELOPMENTAL RATE

Differences in egg size and volume were found between the three river stocks (Table 2). Egg diameter and egg volume were significantly different between the three rivers with the ranking Alta > Allier > Laxa respectively. Alta and Allier eggs were heavier than Laxa eggs and the Alta eggs tended to be heavier than those from the Allier but this was not significant at the 5% level. Previous work on salmonid fish has shown that egg size is related to the age of the female with older fish producing larger eggs in terms of egg diameter, egg weight or size of yolk reserves (Kazakov 1981; Beacham & Murray 1985). Therefore it is likely that the differences in egg size observed in this study are due to the age of the females in each river stock from which the eggs were obtained (Table 1). The eggs from the Laxa river were obtained from 2-3 year old hen fish whilst the Alta and Allier eggs were obtained from females aged 3 years or older.

The effect of temperature on alevin growth, expressed as the increase in body length, from hatching to first feeding for the three rivers is shown in Figure 1. As expected, the rate of development was fastest at 12.5°C, intermediate at 7°C and slowest at 2.5°C. The times taken from hatching to first feeding were; 28, 51 and 124 days for the Laxa fish; 28, 56 and 148 days for the Alta fish; and 29, 43 and 140 days for the Allier fish respectively. Table 2 summarises the mean alevin size at hatching (alevin weight, alevin body length and yolk volume) and at first feeding (fry weight and fry body length) for the Laxa, Alta and Allier fish reared at 12.5, 7.0

Table 1. Geographical source of the Atlantic salmon alevins and the number of families utilised in this study. (MSW = Multi seawinter salmon: 2+ years at sea).

	LAXA (66°N)	ALTA (70°N)	ALLIER (45°30'N)
Females	MSW salmon (5-9 kg)	MSW salmon (101 ± 8 cm)	Landlocked (4+) (450 g)
Males	Grilse/MSW (2-3, 6-12kg)	MSW salmon (94 ± 26) cm	Wild adults
No of Families	30	5	Unknown

Table 2. (a) Weight (W, mg), diameter (d, mm) and volume (V, mm^3) of the eggs from the Laxa (Iceland), Alta (Norway) and Allier (France). (b) Wet weight (mg), total length (mm) and yolk volume (mm^3) at hatching (W_0, TL_0, V_0), wet weight (mg), total length (mm) at first feeding (W_{FF}, TL_{FF}) and length-specific growth rate (SGR_L, mm/day) for Atlantic salmon alevins from the Laxa (Iceland), Alta (Norway) and Allier (France).

		LAXA	ALTA	ALLIER
(a) Egg				
	W	78.9 (1.9)^{a}	149.8 (1.1)^{b}	129.7 (1.1)^{b}
	d	5.0 (0.1)^{a}	6.4 (0.1)^{b}	5.7 (0.2)^{c}
	V	64.4 (1.2)^{a}	135.4 (1.6)^{b}	97.0m(0.2)^{c}
(b) Alevin				
W_0	12.5°C ^{1}	59.5 (2.2)^{a}	^{1}124.2 (1.6)^{b}	^{1}105.7 (4.9)^{c}
	7.0°C	^{1}65.0 (2.1)^{a}	^{1}123.0 (1.7)^{b}	^{1}106.1 (3.2)^{c}
	2.5°C	^{1}66.0 (1.6)^{a}	^{1}124.3 (1.8)^{b}	^{1}104.6 (2.6)^{c}
TL_0	12.5°C	^{1}16.5 (0.1)^{a}	^{1}17.4 (0.2)^{b}	^{1}18.1 (0.2)^{c}
	7.0°C	^{1}16.2 (0.1)^{a}	^{2}18.0 (0.1)^{b}	^{2}18.6 (0.2)^{c}
	2.5°C	^{2}18.0 (0.1)^{a}	^{3}19.2 (0.1)^{b}	^{3}18.7 (0.2)^{c}
V_0	12.5°C	^{1}71.8 (3.8)^{a}	^{1}142.4 (5.6)^{b}	^{1}115.7 (8.8)^{c}
	7.0°C	^{1}69.4 (3.5)^{a}	^{1}141.9 (3.4)^{b}	^{1}92.9 (4.4)^{c}
	2.5°C	^{2}57.4 (2.9)^{a}	^{1}134.1 (5.4)^{b}	^{1}105.9 (5.2)^{c}
W_{FF}	12.5°C	^{1}88.7 (3.2)^{a}	^{1}170.5 (2.0)^{b}	^{1}139.3 (4.5)^{c}
	7.0°C	^{1}91.5 (3.2)^{a}	^{1,2}174.1 (2.6)^{b}	^{1}144.1 (5.6)^{c}
	2.5°C	^{2}101.9 (2.2)^{a}	^{2}183.5 (3.9)^{b}	^{1}151.0 (5.0)^{c}
TL_{FF}	12.5°C	^{1}24.0 (0.2)^{a}	^{1}28.4 (0.2)^{b}	^{1}27.1 (0.4)^{a,b}
	7.0°C	^{1,2}25.3 (0.3)^{a}	^{1,2}28.7 (0.2)^{b}	^{1,2}27.5 (0.2)^{c}
	2.5°C	^{2}26.5 (1.1)^{a}	^{2}29.7 (0.5)^{b}	^{2}28.5 (0.3)^{c}
SGR_L	12.5°C	1.3	1.8	1.4
	7.0°C	0.8	0.8	0.9
	2.5°C	0.3	0.3	0.3

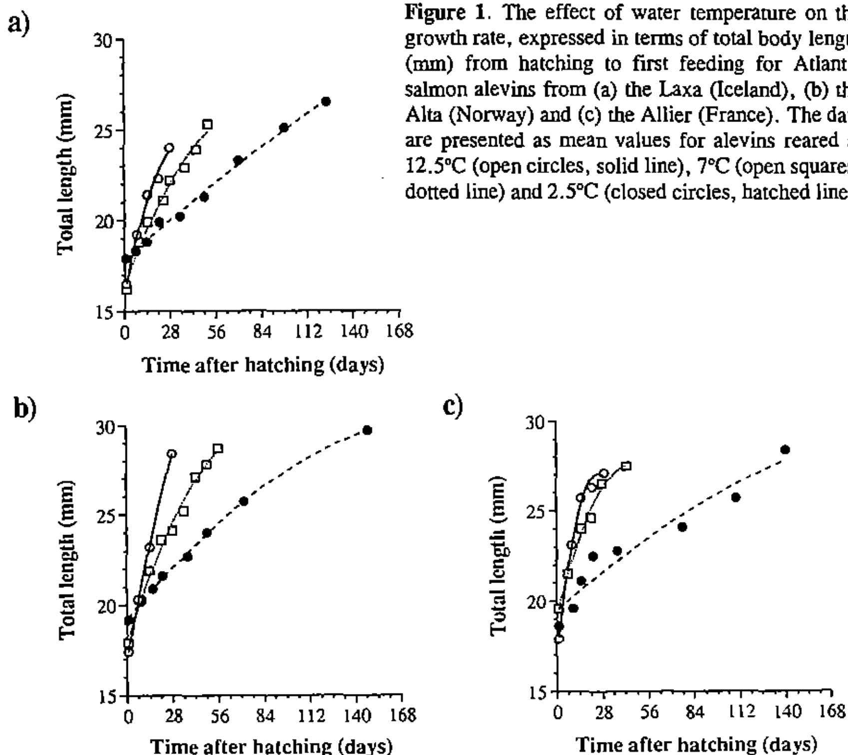

Figure 1. The effect of water temperature on the growth rate, expressed in terms of total body length (mm) from hatching to first feeding for Atlantic salmon alevins from (a) the Laxa (Iceland), (b) the Alta (Norway) and (c) the Allier (France). The data are presented as mean values for alevins reared at 12.5°C (open circles, solid line), 7°C (open squares, dotted line) and 2.5°C (closed circles, hatched line).

and 2.5°C. Statistical comparisons were made comparing 1) fish from the same river stock reared at the three incubation temperatures and 2) fish from the three rivers reared at the same water temperature. These analyses are indicated by the number and letter superscripts respectively in Table 2. The effect of water temperature on alevin size at hatching and first feeding showed a similar pattern for all three river stocks. The total alevin weight (body + yolk) at hatching was similar at the three water temperatures however, alevin body length tended to decrease with increasing water temperature (Table 2). There were no statistical differences in the mean yolk volume at hatching at the three water temperatures. At first feeding, fry wet weight and body length tended to decrease with increasing water temperature (Table 2). Similar temperature-dependent differences in developmental rate and size at first feeding have been reported for a number of salmonid species; Atlantic salmon (Petersen *et al.* 1977, Petersen & Martin-Robichaud 1995), Arctic charr (Wallace & Aasjord 1984), chinook salmon (Heming & McInerney 1982), chum salmon (Beacham & Murray 1985) and steelhead trout (Rombough 1988). At hatching and first feeding a consistent pattern of differences in size were evident between the three river stocks when comparisons were made at either 12.5, 7.0 or 2.5°C (Table 2). Fish from the Alta were significantly larger at hatching and at first feeding compared to the Laxa and Allier fish, and Allier fish were significantly larger at hatching and at first feeding compared to the Laxa fish. These differences in alevin size at hatching and first feeding between fish from the three river stocks reared at the same water temperature were a reflection of initial differences in egg size and yolk volume (Beacham & Murray 1985). However, at each water temperature the growth rates of alevins from the three river stocks appeared to be similar when expressed as the percentage increase in body length per day (Table 2).

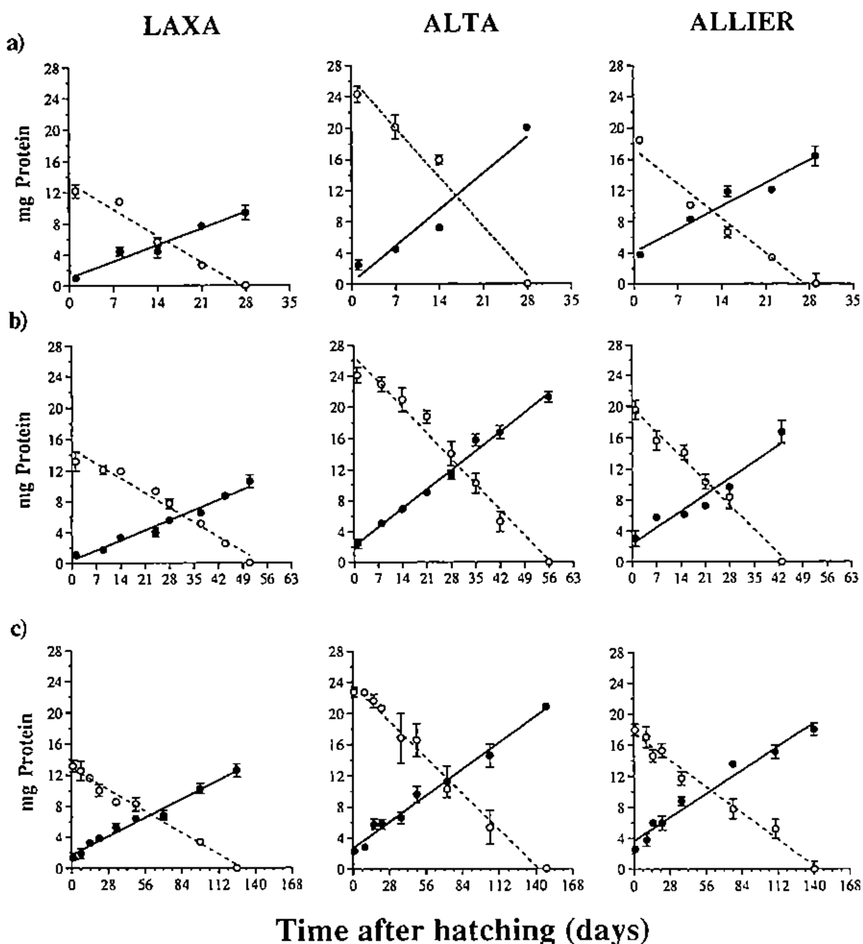

Figure 2. The effect of water temperature on changes in body (closed circles, solid line) and yolk (open circles, dotted line) total protein content at various time intervals from hatching to first feeding at a) 12.5°C, b) 7.0°C and c) 2.5°C for Atlantic salmon alevins from the Laxa (Iceland), Alta (Norway) and Allier (France). The data are presented as mean values ± SEM (n=20).

2) NITROGEN BALANCE AND METABOLIC RATE

Significant linear relationships were found between time from hatching to first feeding and the decrease in yolk protein content and increase in body protein content for the Laxa, Alta and Allier alevins reared at 12.5, 7.0 and 2.5°C (Figure 2, regression analysis not shown). For each river, there was a significant decrease in both the rate of yolk protein loss (mg protein/day) and the rate of body protein gain (mg protein/day) with decreasing water temperature (ANCOVA, data not shown). At each incubation temperature, significant differences were found in the rates of yolk protein loss and body protein gain (mg protein/day), between hatching and first feeding between the three rivers with the ranking Alta > Allier > Laxa respectively (ANCOVA, data not shown). Simple nitrogen balances (yolk N loss = body N gain + ammonia excretion) were constructed for the alevins from the Laxa, Alta and Allier at 12.5, 7.0 and 2.5°C (Table 3). The nitrogen content (mg N) of the body and yolk at hatching and body nitrogen content at first feeding were calculated by dividing the protein content by 5.85 (Gnaiger & Bitterlich, 1984). The yolk nitrogen loss and body nitrogen gain (mg N/day) for each group was estimated as the slope of the regression line relating the changes in the body and yolk nitrogen content (protein/5.85) with time from hatching to first feeding. Gross yolk-nitrogen conversion efficiency was calculated as the ratio of the slope of these two regression lines, ie (body-N growth/yolk-N absorption) x 100 (Dumas *et al.* 1995). The relationship between ammonia excretion and time from hatching to first feeding was linear for the Laxa, Alta and Allier alevins at 12.5, 7.0 and 2.5°C (data not shown) and the slope of each regression line was used as an estimate of daily ammonia excretion (mg NH_3/day). Using this simple model, the mean percentage accountability was 101 $\pm$2.4 % (range 91 - 111 %). A similar temperature-dependent change in nitrogen balance and standard metabolic rate was seen for each river. As water temperature decreased, the rates of yolk nitrogen loss, ammonia excretion, body nitrogen gain and standard metabolic rate decreased (Tables 3 and 4).

However, gross yolk conversion efficiency increased with decreasing water temperature (Table 3), and the average efficiencies at each water temperature were 70.4% (12.5°C), 73.8% (7.0°C) and 83.5% (2.5°C) respectively. There is very little data on protein/nitrogen yolk conversion efficiencies (YCE) in salmonid fish however, efficiencies of 72-74% at 7°C can be calculated for Atlantic salmon from the data of Srivastava & Brown (1991, 1993). The efficiency of yolk utilisation for growth in salmonid alevins has commonly been expressed in terms of changes in dry weight and these data have shown a similar decrease in YCE with increasing water temperature (Heming, 1982; Wallace & Aasjord, 1984; Rombough 1988). A significant correlation was found between yolk conversion efficiency and standard metabolic rate (Figure 3). Standard metabolic rate decreased with decreasing water temperature resulting in an increase in yolk nitrogen available for growth and an increase in yolk conversion efficiency.

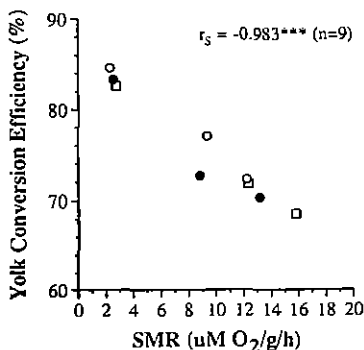

Figure 3. The relationship between standard metabolic rate (μM O_2/g/h) and yolk-N conversion efficiency (Body-N gain*100/yolk-N loss) for Atlantic salmon alevins from (a) the Laxa (Iceland, closed circles), (b) the Alta (Norway, open circles) and (c) the Allier (France, open squares). The Spearman rank correlation coefficient (r_s) is indicated (*** $p < 0.001$).

Table 3. Effect of temperature on nitrogen balance in Atlantic salmon alevins from the Laxa (Iceland), Alta (Norway) and Allier (France). The nitrogen content the alevin at hatching (B_0, Y_0) and first feeding (B_{FF}) is expressed as mg of body or yolk nitrogen, the rates of ammonia excretion, yolk nitrogen loss and body nitrogen gain are expressed as mg nitrogen/day. Yolk conversion efficiency (YCE, %) is calculated as $[(B_{FF}-B_0)*100]/Y_0$.

T°C	12.5	7.0	2.5
a) LAXA			
$Body_0$	0.171	0.188	0.239
$Yolk_0$	2.085	2.256	2.256
$Body_{FF}$	1.607	1.812	2.137
Body N gain	0.052	0.032	0.015
Yolk N loss	-0.074	-0.044	-0.018
NH_3 excretion	0.021	0.015	0.005
YCE	70.3	72.7	83.3
b) ALTA			
$Body_0$	0.410	0.342	0.393
$Yolk_0$	4.154	3.419	3.880
$Body_{FF}$	3.419	3.077	3.556
Body N gain	0.107	0.047	0.022
Yolk N loss	-0.148	-0.061	-0.026
NH_3 excretion	0.031	0.016	0.006
YCE	72.3	77.0	84.6
c) ALLIER			
$Body_0$	0.650	0.530	0.444
$Yolk_0$	3.145	3.350	3.077
$Body_{FF}$	2.803	2.957	3.077
Body N gain	0.074	0.056	0.019
Yolk N loss	-0.108	-0.078	-0.023
NH_3 excretion	0.026	0.015	0.005
YCE	68.5	71.8	82.6

Table 4. Standard metabolic rates ($\mu M\ O_2/g/h$) at first feeding for Atlantic salmon fry from the Laxa (Iceland), Alta (Norway) and Allier (France) rivers reared at 2.5, 7.0 and 12.5°C.

	LAXA	ALTA	ALLIER
12.5°C	[1]13.18 (0.66)[a,b]	[1]12.24 (0.81)[a]	[1]15.79 (0.92)[b]
7.0°C	[2] 8.82 (0.34)[a]	[2] 9.39 (0.26)[a]	[1]12.35 (0.24)[b]
2.5°C	[3] 2.55 (0.33)[a]	[3] 2.31 (0.27)[a]	[2] 2.73 (0.27)[a]

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REFERENCES

Beacham, T.D. & Murray, C.B. (1985). effect of female size, egg size and water temperature on developmental biology of chum salmon (*Oncorhynchus keta*) from the Nitinat River, British Columbia. *Canadian Journal of Fisheries and Aquatic Sciences*, **42**, 1755-1765

Blaxter, J.H.S. (1992). The effect of temperature on larval fishes. *Netherlands Journal of Zoology*, **42**, 336-357.

Brännäs, E. (1988). Emergence of Baltic salmon, *Salmo salar* L., in relation to temperature: a laboratory study. *Journal of Fish Biology*, **33**, 589-600.

Crisp, D.T. (1988). Prediction, from temperature, of eying, hatching and 'swim-up' times for salmonid embryos. *Freshwater Biology*, **19**, 41-48

Dumas, S., Blanc, J.M, Audet, C. & de la Noüe, J. (1995). Variation in yolk absorption and early growth of brook charr, *Salvelinus fontinalis* (Mitchill), Arctic charr, *Salvelinus alpinus* (L.), and their hybrids. *Aquaculture Research*, **26**, 759-764.

Gnaiger, E. & Bitterlich, G. (1984). Proximate biochemical composition and caloric content calculated from elemental CHN analysis: stoichiometric concepts. *Oecologia (Berlin)*, **62**, 289-298.

Gunnes, K. (1979). Survival and development of Atlantic salmon eggs and fry at three different temperatures. *Aquaculture*, **16**, 211-218.

Hayes, F.R. & Pelluet, D. (1945). The effect of temperature on the growth and efficiency of yolk conversion in the salmon embryo. *Canadian Journal of Research*, **23**, 7-15.

Heming, T.A. (1982). Effects of temperature on utilisation of yolk by chinook salmon (*Oncorhynchus tshawytscha*) eggs and alevins. *Canadian Journal of Fisheries and Aquatic Sciences*, **39**, 184-190.

Heming, T.A. & McInerney, J.E. (1982). Effect of temperature on initial feeding in alevins of chinook salmon (*Oncorhynchus tshawytscha*). *Canadian Journal of Fisheries and Aquatic Sciences*, **39**, 1554-1562.

Jensen, A.J., Johnsen, B.O. & Saksgård, L. (1989). Temperature requirements in Atlantic salmon (*Salmo salar*), Brown trout (*Salmo trutta*) and Arctic charr (*Salvelinus alpinus*) from hatching to initial feeding compared with geographic distribution. *Canadian Journal of Fisheries and Aquatic Sciences*, **46**, 786-789.

Kane, T.R. (1988). Relationship of temperature and time of initial feeding of atlantic salmon. *Progressive Fish Culturist*, **50**, 93-97.

Kazakov, R.V. (1981). The effect of size of Atlantic salmon, *Salmo salar* L., eggs on embryos and alevins. *Journal of Fish Biology*, **19**, 353-360.

Pannevis, M.C. & Houlihan, D.F. (1992). The energetic cost of protein synthesis in isolated hepatocytes of rainbow trout (*Oncorhynchus mykiss*). *Journal of Comparative Physiology B*, **162**, 393-400.

Petersen, R.H. & Martin-Robichaud, D.J. (1995). Yolk utilization by Atlantic salmon (*Salmo salar* L.) alevins in response to temperature and substrate. *Aquacultural Engineering*, **14**, 85-99.

Petersen, R.H., Spinney, H.C.E. & Sreedharan, A. (1977). Development of atlantic salmon (*Salmo salar*) eggs and alevins under varied temperature regimes. *Journal of the Fisheries Research Board of Canada*, **34**, 31-43.

Rombough, P.J. (1988). Growth, aerobic metabolism, and dissolved oxygen requirements of embryos and alevins of steelhead, *Salmo gairdneri*. *Canadian Journal of Zoology*, **66**, 651-660.

Rombough, P.J. (1994). Energy partitioning during fish development: additive or compensatory allocation of energy to support growth?. *Functional Ecology*, **8**, 178-186.

Rombough, P.J. (1996). The effects of temperature on embryonic and larval development. In *Global Warming - Implications for Freshwater and Marine Fish* (eds. McDonald, D.G. & Wood, C.M.) In press. Cambridge: Cambridge University Press.

Srivastava, R.K. & Brown, J.A. (1991). The biochemical characteristics and hatching performance of cultured and wild Atlantic salmon eggs. *Canadian Journal of Zoology*, **69**, 2436-2441.

Srivastava, R.K. & Brown, J.A. (1993). assessment of egg quality in Atlantic salmon, *Salmo salar*, treated with testosterone: biochemical composition. *Canadian Journal of Zoology*, **70**, 109-115.

Wallace, J.C. & Aasjord, D. (1984). The initial feeding of arctic charr (*Salvelinus alpinus*) alevins at different temperatures and under different feeding regimes. *Aquaculture*, **38**, 19-33.

SENSITIVITY TO MECHANICAL SHOCK IN ATLANTIC SALMON EGGS DURING THEIR FIRST SIX HOURS AFTER FERTILIZATION

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Abstract

Atlantic salmon eggs from six domestic female and six sea-run kelts were tested for mechanical shock sensitivity from 0.5 to 6.0 hours after fertilization using force generated by dropping eggs from measured heights. Estimates of drop height and force causing 10 (LC10) and 25% (LC25) mortality were used to compare sensitivity to shock relative to handling encountered in the process of collecting, disinfecting, and transporting of eggs to incubation facilities. There were no significant differences in LC10 and LC25 estimates among sample times through 6 hours post-fertilization. Estimates of shock causing 10% mortality ranged from drop heights of 23.5 to 26.9 cm or force of 5.2 to 6.0 X 10^3 ergs for domestic eggs and 17.5 to 38.5 cm or 3.5 to 7.7 x 10^3 ergs for kelt eggs. LC25 estimates of drop height and force were 46.1 to 60.3 cm and 10.2 to 13.4 x 10^3 ergs for domestic stock and 43.7 to 65.1 cm and 8.8 to 13.1 x 10^3 ergs for kelt eggs. Variability in shock sensitivity among females was high, and similar to differences in 24-hour mortality found in eggs transported for incubation.

Introduction

Atlantic salmon (*Salmo salar*) egg mortality has increased for eggs collected, fertilized, and then transported to incubation facilities located several hours travel from the fertilization site. Once fertilized, eggs require handling and receive shocks from procedures like disinfection, packing, transport, unpacking, a second disinfection, and placement into incubators. Once all procedures are completed and eggs are transported to incubation facilities, significant mortality occurs the first day after fertilization. The mortality rates are highly variable among eggs from individual females. The purpose of this and a related study is to determine the major causes of egg mortality during transport in Atlantic salmon.

In general, salmonid eggs are thought to withstand shock from routine handling for the first 48 hours after fertilization (Piper et al. 1982). We determined the effects of mechanical shock to eggs during the course of typical transport times used in the Atlantic salmon egg program, apart from possible effects of transport shock. We also tested two types of Atlantic salmon broodstock (domestic stock and reconditioned wild adults, or kelts) for variability between groups and among individuals. The methods used were intended to simulate shock to eggs from water poured over eggs during disinfection or packing procedures. The mechanical shock administered by dropping eggs was to approximate the effect of pouring eggs in water from a similar height (Jensen and Alderdice 1983). We use mechanical shock treatments similar to those of Jensen and Alderdice (1983, 1989) to test differences in shock sensitivity of eggs during the first 6 hours after fertilization.

Methods and Materials

Eggs were collected from six individuals of domestic strain Atlantic salmon (Cronin National Salmon Station, Sunderland, Massachusetts) and six kelts (North Attleboro National Fish Hatchery, North Attleboro, Massachusetts). After fertilization in 10° C water, samples of 20 to 45 eggs were placed into 500 ml jars to serve as controls and for the set of 30-minute sample jars. One set, a negative control, was placed into coolers and not handled further; the other control set was handled the same as those given mechanical shock, but not shocked. At each sampling period thereafter (1, 2, 4, and 6 hours post-fertilization) one control group of eggs was placed into jars and three test (mechanical shock) groups were also placed into separate jars. A pre-test showed that drop heights of 10, 40, and 90 cm produced low, medium, and high egg mortality, leading to selection of those heights for the tests.

To start a test, eggs were gently removed from sample jars, and placed into 6-cm-diameter petri dishes. After all water was drained, dishes were either dropped the appropriate height (experimental groups) or placed back into jars without further handling (controls) as in Jensen and Alderdice (1983, 1989). After a single shock challenge, eggs were transferred back to the water-filled sample jars and placed into coolers for storage at 6-8° C until the end of the test. Two hours after completion of the final mechanical shock, water was drained from jars and eggs placed either into Davidson's fixative (six parts ethanol, four parts formalin, two parts acetic acid and six parts distilled water; for the domestic broodstock eggs) or 10% formalin (kelt eggs). Eggs fixed in Davidson's fixative were examined under 7X magnification to determine mortality because fixed dead eggs returned to amber color. Dead eggs in formalin were easily identified without magnification.

Statistical analysis included estimation of force or drop height causing 10 and 25% mortality (LC10 and LC25) using estimates generated through Tablecurve 2D software (Jandel Scientific, San Rafael, California). Force, or energy transferred to the eggs, in 1×10^3 ergs, was calculated as in Jensen and Alderdice (1989). One-way analysis of variance (ANOVA) was used to determine if there were differences in shock sensitivity among the sample times and two-way ANOVA to determine differences between broodstock groups. All statistical tests were conducted at the $p=0.05$ level of significance.

Results

Estimates of 10 and 25% mortality were similar for both domestic and kelt eggs and are shown as LC10 and LC25 as cm drop height, as force estimated to cause mortality, and as 95% confidence intervals of percent mortality at LC10 and LC25 (Table 1). In all cases, variability among estimates was high, because eggs from certain individual females were much more sensitive to shocks than others (Table 1). Force associated with egg mortality estimates (Table 1) was similar among broodstock types and sample times through the 6-hour sample period. Force calculations ranged between 3.5 and 7.7×10^3 ergs for all LC10 estimates, with most estimates between 5.0 and 6.0×10^3 ergs. Force estimates for 25% egg mortality ranged from 8.8 to 13.4×10^3 ergs.

Table 1. Drop height (cm) estimates of 10% (LC10) and 25% (LC25) Atlantic salmon egg mortality (95% confidence intervals) and corresponding force (10^3 ergs) from 0.5 to 6.0 hours after fertilization of mortality for LC10 and LC25.

Broodstock	Hours	LC10 (cm)	Force (10^3 ergs)	Mortality 95% CI	LC25 (cm)	Force (10^3 ergs)	Mortality 95% CI
Domestic	0.5	26.9	6.0	(4.8 to 15.2%)	59.2	13.1	(15.9 to 34.1%)
	1.0	26.9	6.0	(4.8 to 15.2%)	59.2	13.1	(15.9 to 34.1%)
	2.0	23.5	5.2	(4.5 to 15.5%)	56.2	12.5	(16.7 to 33.3%)
	4.0	25.3	5.6	(5.5 to 14.5%)	60.3	13.4	(18.6 to 31.4%)
	6.0	25.2	5.6	(-1.8 to 21.8%)	46.1	10.2	(15.1 to 34.9%)
Kelt	0.5	26.5	5.3	(-1.1 to 21.1%)	53.6	10.8	(13.5 to 36.5%)
	1.0	38.5	7.7	(-0.3 to 20.3%)	65.1	13.1	(-31.3 to 81.3%)
	2.0	17.5	3.5	(-0.3 to 20.3%)	43.7	8.8	(10.7 to 39.3%)
	4.0	25.6	5.1	(1.0 to 19.0%)	52.5	10.6	(13.5 to 36.5%)
	6.0	27.6	5.5	(-9.4 to 29.4%)	48.4	9.7	(3.5 to 46.5%)

Estimates of 10 and 25% mortality in domestic broodstock were nearly the same over 6 hours post-fertilization. Variability in egg mortality among the six individuals ranged from 5 to 20%. More variability in estimates occurred with kelts than domestics; however, mortality rates were similar in the two groups.

Discussion

Atlantic salmon egg mortality measured in this study, and at the water temperatures experienced, considers egg development stages which were was limited to elevation of the blastodisc, before the first cell division, or before the two-celled stage (Battle 1944). Sampling times for Atlantic salmon eggs in this study approximated those used for handling and transport in the current Connecticut River program. The timing of mechanical shock to eggs during this period did not appear to affect egg mortality. Coho salmon (*Oncorhynchus kisutch*) appeared slightly more sensitive to mechanical shock than Atlantic salmon, with a median shock limit of 31.1 cm decreasing to 16.4 cm at 8 hours after fertilization (Jensen and Alderdice 1983). Coho salmon eggs used in the Jensen and Alderdice (1983) study were less sensitive to shock during the first hour after fertilization, as median shock sensitivities ranged from 383.4 to 38.3 cm.

Jensen and Alderdice (1989) tested shock sensitivity of six salmonid species: coho, chinook (*O. tshawytscha*), sockeye (*O. nerka*), chum (*O. keta*), pink salmon (*O. gorbuscha*), and steelhead (*O. mykiss*); they also determined the LC10 and LC50 values for these groups through the incubation period. The LC10 values for Atlantic salmon eggs in our study are comparable to those from inactivated fertilized eggs and stage 1 eggs (before the first cell division), both sensitive periods for eggs of the six species listed above. LC10 shock sensitivity (in drop height) of inactivated fertilized eggs for these species ranged from 12.1 to 41.7 cm. Values for Atlantic salmon were within this range for domestics (26.9 cm) and kelts (26.5 cm). Estimates of force transferred to cause 10% mortality in Atlantic salmon eggs one half hour after fertilization (5.3 to 6.0 x 10^3 ergs) were similar to those of Pacific salmonids (3.9 to 7.0 x 10^3 ergs; Jensen and Alderdice 1989), with the exception of a lower estimate (1.2 x 10^3 ergs) for sockeye salmon. Sensitivity of eggs shocked between 1 and 6 hours compared favorably with eggs from Jensen and Alderdice

(1989) stage 1, or mounded single cell cytoplasm. Of the Pacific salmonids, only sockeye had a shock sensitivity close to Atlantic salmon (23.0 cm versus 23.5 to 38.5 cm). All other salmonids were somewhat more sensitive to shock during this period than Atlantic salmon, with steelhead being most sensitive (LC10 of 8.2 cm) and chinook least sensitive (18.2 cm). Jensen and Alderdice (1989) found breakage in coho and steelhead eggs at lower force (1.2 to 1.9×10^3 ergs) than that for Atlantic salmon which was LC10 from 3.5 to 7.7×10^3 ergs. Other Pacific salmonid species had similar force estimates at LC10 (Jensen and Alderdice 1989). Considering the variability in design of tests run with Atlantic salmon, these estimates are probably not different.

Variability in LC estimates was high in our Atlantic salmon study because samples were not pooled, but rather tested as individual female groups. Similar variability is present in 24-hour egg mortality data among Atlantic salmon egg groups (W. F. Krise, unpublished data). Jensen and Alderdice (1983) subjected coho salmon eggs to mechanical shock and found that breakage force of eggs was similar between 0 and 100 cm whether dropped or poured. The current program for Atlantic salmon egg incubation in northeastern states usually requires handling and transport of eggs for 4 hours or more. Most egg mortality probably occurs during handling procedures, because handling includes pouring eggs on several occasions for disinfection, packing, or unpacking. Further research is underway to determine benefits of delayed fertilization after completion of transport. Additional research should be conducted to determine (1) effects of transport after fertilization, (2) average estimates of shock sensitivity using replicate egg groups pooled from several adults, and (3) development of reduced shock handling procedures.

References

Battle, HI 1944 The embryology of the Atlantic salmon (*Salmo salar*, Linnaeus). Canadian Journal of Research 22:105-125.

Jensen, JOT, and Alderdice, DF 1983 Changes in mechanical shock sensitivity of coho salmon (*Oncorhynchus kisutch*) eggs during incubation. Aquaculture 32:303-312.

Jensen, JOT, and Alderdice, DF 1989 Comparison of mechanical shock sensitivity of eggs of five Pacific salmon (*Oncorhynchus*) species and steelhead trout (*Salmo gairdneri*). Aquaculture 78:163-181.

Piper, RG, McElwain, IB, Orme, LE, McCraren, JP, Fowler, LG, and Leonard, JR 1982 Fish Hatchery Management. United States Department of the Interior, Fish and Wildlife Service, Washington, DC.

EARLY LIFE-STAGE OUTBREAKS OF SYSTEMIC BACTERIAL COLD-WATER DISEASE - IS THE CAUSAL AGENT *FLEXIBACTER PSYCHROPHILUS* VERTICALLY TRANSMITTED IN SALMONIDS?

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Abstract

Anecdotal data and early lab tests indicated that *Flexibacter psychrophilus* was responsible for high losses in early life stages of steelhead trout in a California hatchery and further, that the pathogen may be vertically transmitted. To determine the source of infections, eggs and embryos from 17 steelhead trout were taken at selected developmental stages. Some of the broodstock steelhead trout used for this study had been injected with erythromycin prior to spawning. Others had been injected with oxytetracycline, others had not been injected. The eggs/embryos were incubated in TYE broth and determined to be surface-disinfected when no bacterial growth was isolated from the broth after 72 h incubation at 17 °C. Surface-disinfected eggs/embryos were then homogenized and cultured in TYE and growth was identified as *F. psychrophilus* by biochemical and immunological assays. A source of surface *F. psychrophilus* contamination was the hatchery water, infecting the surface of 29% of eggs/embryos. *F. psychrophilus* was also detected within the ovarian fluid samples of 10% of the broodstock trout. *F. psychrophilus* was isolated from the contents of 13% of newly spawned eggs, as well as from 7% of eyed eggs and from 4% of newly hatched alevins. There was no difference observed in the proportion of eggs infected with *F. psychrophilus* from antibiotic-injected versus non-injected fish. However, the mortalities were monitored in the remaining progeny of the experimental fish and the progeny if the broodstock injected with erythromycin experienced significantly lower mortalities due to coldwater disease. *In vitro* experiments indicate that *F. psychrophilus* is somewhat resistant to lysozyme concentrations of up to 2 mg ml^{-1} (greater than concentrations found within a salmonid egg). The results of this study indicate that *F. psychrophilus* may be transmitted both horizontally and vertically within salmonid hatcheries.

Introduction

High mortalities due to systemic bacterial cold-water disease (BCWD) were documented in steelhead trout (*Oncorhynchus mykiss*) at a hatchery in Northern California. Losses up to 85% during the first two months of rearing were observed in some lots of fish. All steelhead lots developed systemic BCWD within 1 to 4 weeks of button-up. The eggs and young fish had been

incubated in sand filtered spring water, which is fish-free. Yellow colonies typical of *Flexibacter psychrophilus,* causal agent of BCWD, were isolated on medium inoculated with surface disinfected steelhead egg homogenates. In the light of these anecdotal data and early tests, the study documented here was initiated, the purpose of which was to determine if *F. psychrophilus* could be transmitted within steelhead eggs, and if this was the route of the observed infections due to *F. psychrophilus.* Another objective of this study was to determine if injecting antibiotics into broodstock female steelhead would reduce the prevalence of intra-ovum infections (if any) due to *F. psychrophilus.*

Materials and Methods

Two stocks of steelhead trout were examined. Five female fish from the Scott Creek (SC) stock were injected with erythromcyin (20 mg kg^{-1} fish weight) upon receipt and at 30 d intervals thereafter. Seven females from the San Lorenzo (SL) stock were injected with tetracycline (20 mg kg^{-1} fish weight), following the same regime. Five more SL females were left uninjected. At spawning samples of eggs were taken from each fish and transported to the laboratory in their own coelomic fluid. Additional eggs were sampled from each fish after the eggs had been fertilized, surface disinfected and water-hardened with 100 ppm povidone/iodine for 1h, and then rinsed in the hatchery water. Additional samples were taken from each fish at the eyed and hatch stage.

To examine the eggs/embryos for intra-ovum infections due to *F. psychrophilus* we followed a modified protocol of Evelyn *et al.* (1984). In the laboratory, 5 unfertilized, non water-hardened eggs were blotted on sterile filter paper and then placed in individual tubes containing 3 ml of tryptone-yeast extract broth that had been supplemented with 0.5% (v/v) newborn calf serum (TYE). In the case of the surface disinfected eggs, eyed eggs, and newly hatched sac fry, 5 eggs/embryos were treated as above, 5 additional eggs/embryos from each sample were surface disinfected again with 400 ppm povidone/iodine for 15 minutes, after which they were rinsed 5 times with sterile distilled water. The disinfected eggs/embryos were placed in TYE broth as above. All TYE tubes were incubated at 17 °C for 72h and then examined for turbidity. The TYE broth from each tube was streaked onto TYE plates and those plates were then incubated at 17 °C for 72h.

In order to determine if *F. psychrophilus* was transmitted within the steelhead eggs, the following was done. If the TYE broth in a given tube was clear and free of turbidity, the egg/embryo was crushed and homogenized with a sterile glass rod, and the homogenate was then incubated at 17 °C for an additional 72h, after which the homogenate was streaked onto TYE plates and the plate incubated at 17 °C for an additional 72h.

To examine the possibility that *F. psychrophilus* could survive inside salmonid eggs, the contents of 50 steelhead eggs from the uninjected SL group were taken aseptically with 1 ml syringes and disposable 18 ga. needles. The contents of 5 eggs were pooled into sterile Eppendorf tubes, so that there were 10 pooled samples in total. The egg contents were then spiked with *ca.* 10 *F. psychrophilus* cells. The tubes were then incubated at 17 °C for 7d. The egg contents were then streaked onto TYE plates and the plates were then incubated at 17 °C for 72h. Any growth was confirmed as being due to *F. psychrophilus* according to the criteria outlined above.

All growth on all plates was examined to determine if it was due to *F. psychrophilus.* Growth was deemed to be positive if it met the following criteria: Gram negative short rods, producing yellow pigment on TYE medium, that the yellow pigment turned red when streaked onto filter paper soaked in 1N NaOH (indicative of flexirubin, the pigment produced by *Flexibacter spp.*),

growth at 17 °C, but not at 30 °C, no growth when the TYE broth (before homogenization) was streaked onto TYE plates, and a positive slide agglutination result. The slide agglutination was done with a saline suspension of the growth to be examined, and antisera raised against *F. psychrophilus* in rabbits. The antisera was kindly supplied by Dr. R. Hedrick (Department of Veterinary Medicine, University of California at Davis).

To determine whether *F. psychrophilus* is resistant or susceptible to lysozyme, which is found in salmonid eggs and is probably responsible for passive defense of the developing embryos against bacterial pathogens, we followed a modification of the procedure described by Yousif *et al.* (1994). A suspension of *F. psychrophilus* cells was made in phosphate-buffered saline (PBS) and adjusted to an absorbance of *ca.* 10.0 at 540 nm. We also suspended *Aeromonas salmonicida* (an A+ strain) cells in the same way, as a positive control for lysozyme activity. The bacteria (both *F. psychrophilus* and *A. salmonicida*) were then diluted 1/10 in solutions of hen egg white lysozyme in PBS (pH 6.2), at concentrations of 0, 0.1, 1.0, and 2.0 mg ml^{-1}. Samples of bacteria in each lysozyme concentration were taken at 0, 30, 60, and 90 minutes. The cells were washed once in PBS and then serially diluted 100-fold to 10^{-6}. 25 µl of each diluted sample were dropped, in triplicate, onto TYE plates. The plates were then incubated at 17 °C for 48h, after which colonies were counted. Results are expressed as the percentage reduction in cell number, using the colony counts from the control tubes (0 mg ml^{-1} lysozyme) as the standard (0%) reduction.

At the outset of this study it became apparent that *F. psychrophilus* was contaminating the surface of some of the eggs/embryos (see **Results and Discussion**), despite iodine disinfection procedures at the hatchery. In order to determine the susceptibility of *F. psychrophilus* to povidone the above experiment was repeated, except that the bacterial suspensions (both *F. psychrophilus* and *A. salmonicida*) were exposed to povidone/iodine concentrations of 0, 10, 100, and 500 ppm in sterile, distilled water. The procedure was as above, except that samples were taken at 0, 30 and 60 minutes only.

The remaining progeny of the 17 experimental fish were reared in the hatchery according to standard hatchery practice and mortalities were monitored. Any mortalities were determined to be due to BCWD by culture and immunoassays, in addition to noting characteristic pathological signs and direct observation of typical *Flexibacter spp.* cells from spleen squashes examined at 600 - 1000x by phase microscopy.

Results and Discussion

A source of infection due to *F. psychrophilus* was the hatchery water. Thirty percent of all of the newly spawned and fertilized eggs, eyed eggs, and yolk sac fry were surface contaminated with *F. psychrophilus* (Table 1). This was despite the fact that the newly spawned eggs had been surface disinfected with povidone/iodine at the hatchery. This may be a problem unique to this particular hatchery, i.e., the bacteria in the water infect the surface of eggs/embryos after the initial disinfection. We also isolated *F. psychrophilus* from the surface of 10% of newly spawned eggs that had only been in contact with ovarian fluid from the spawning female, indicating that the females themselves were the source of infection in these cases. Therefore, complete surface disinfection is essential, especially in hatcheries with pathogen-free water. The iodine/povidone experiment indicated that *F. psychrophilus* is not resistant to iodine at 100 ppm (Table 2).

Table 1. Percentage (%) of eggs or embryos positive for *Flexibacter psychrophilus*. SC = Scott creek, SL = San Lorenzo, Erythro. = erythromycin, Oxytet. = oxytetracycline, Not - not injected

Fish #	Stock	Injected	Surface	Ovarian	Egg	Eyed	Alevin
1	SC	Erythro.	47	15	15	0	0
2	SC	Erythro.	27	0	n.d.	20	0
3	SC	Erythro.	20	10	50	20	40
4	SC	Erythro.	9	0	10	0	0
5	SC	Erythro.	14	20	0	20	0
Average			23	9	15	12	8
6	SL	Oxytet.	53	0	10	0	0
7	SL	Oxytet.	47	0	10	0	0
8	SL	Oxytet.	47	0	10	15	10
9	SL	Oxytet.	33	30	10	0	0
10	SL	Oxytet.	10	0	0	10	n.d.
11	SL	Oxytet.	40	20	30	10	n.d.
12	SL	Oxytet.	0	30	10	20	0
Average			33	12	12	8	2
13	SL	Not	20	10	0	0	0
14	SL	Not	0	10	15	0	0
15	SL	Not	53	10	30	n.d.	10
16	SL	Not	33	10	0	0	0
17	SL	Not	33	10	10	0	0
Average			28	10	11	0	2
Overall average			29	**10**	13	7	4

Table 2. Susceptibility of *Flexibacter psychrophilus* and *Aeromonas salmonicida* to povidone/iodine

Bacterial species	Iodine concentration (ppm)	% Reduction of cfu after exposure of t= 0 min	30 min	60 min
Aeromonas salmonicida				
	0	0	0	0
	10	0	100	100
	100	0	100	100
	500	0	100	100
Flexibacter psychrophilus				
	0	0	0	0
	10	0	50	100
	100	0	98	100
	500	0	100	100

F. psychrophilus was isolated from the contents of 13% of newly spawned eggs, as well as from 7% of eyed eggs and from 4% of newly hatched alevins (Table 1). Previously it was thought that *Renibacterium salmoninarum*, causal agent of bacterial kidney disease, may be the only bacterial pathogen of salmonids that could survive within salmonid eggs (Evelyn *et al.* 1984, Barker *et al.* 1991, Yousif *et al.* 1994) Most other bacterial salmonid pathogens are Gram negative, including *F. psychrophilus*. Susceptibility to lysozyme is a characteristic of many fish pathogens (Grinde 1989), and Yousif *et al.* (1994) have shown that a number of Gram negative fish pathogens are susceptible to lysozyme purified from coho salmon (*O. kisutch*) eggs. However, those authors did not test *F. psychrophilus* for lysozyme susceptibility. Our *in vitro* experiments for lysozyme susceptibility indicate that *F. psychrophilus* is somewhat resistant to lysozyme. Exposure of *F. psychrophilus* to 2 mg ml^{-1} for 90 minutes resulted in only a 44% reduction in the number of viable cells, as compared to a 99% reduction in *Aeromonas salmonicida* viability when *A. salmonicida* was exposed under the same conditions (Table 3). These data were supported by the fact that *F. psychrophilus* was isolated from 100% of the samples of egg contents that had been spiked with *F. psychrophilus* cells. It would seem that *F. psychrophilus* is somewhat resistant to the defense systems that are present within salmonid eggs.

Table 3. Susceptibility of *Flexibacter psychrophilus* and *Aeromonas salmonicida* to hen egg white lysozyme

		% Reduction of cfu after exposure of t=			
Bacterial species	Lysozyme concentration (mg ml^{-1})	0 min	30 min	60 min	90 min
Aeromonas salmonicida					
	0.0	0	0	0	0
	0.1	1	56	74	53
	1.0	2	56	78	67
	2.0	1	99	98	99
Flexibacter psychrophilus					
	0.0	0	0	0	0
	0.1	0	20	0	31
	1.0	0	10	60	46
	2.0	0	30	55	44

There was no significant difference in the prevalence of intra-ovum infection due to *F. psychrophilus* within eggs and embryos from antibiotic injected, versus non-injected broodstock (Table 1). This was the case with both the Scott Creek (injected with erythromycin) and San Lorenzo (approximately half injected with tetracycline) stocks. Nor was there any significant difference in the prevalence of surface contamination due to *F. psychrophilus* within the ovarian fluid of antibiotic injected versus non-injected female broodstock (Table 1). However, there were significantly fewer mortalities due to BCWD within the Scott Creek stock than the San Lorenzo stock (Table 4). The Scott Creek broodstock had been injected with erythromycin before spawning.

Table 4. Mortalities due to BCWD within progeny of antibiotic-injected versus non-injected salmonids, SH = Steelhead, C = Coho, SC = Scott Creek, SLR = San Lorenzo River, E = Erythromycin, O = Oxytetracycline, N = Not injected

			- Average Daily Mortality (%) -				
Stock	n	Inj.	Week 1	Week 2	Week 3	Week 4	Proj. Ann. Mort. (%)
SH-SC	7629	E	0.01	0.10	0.07	0.13	28.3
SH-SC	8729	E	0.16	0.06	0.05	0.08	31.9
SH-SLR	8648	O	0.13	0.54	1.05	1.56	299.3
SH-SLR	8818	O	0.12	0.44	1.00	1.75	302.0
SH-SLR	9630	N	0.11	0.26	0.87	2.21	314.8
SH-SLR	6348	N	0.13	0.28	0.28	0.45	104.0
C-SC	8184	E	0.07	0.07	0.04	0.03	19.2
C-SC	8188	E	0.05	0.06	0.10	0.03	21.9
C-SC	7795	E	0.03	0.07	0.05	0.04	17.3

It should be pointed out that the *F. psychrophilus* isolated from the contents of the newly spawned eggs, and eyed eggs (Table 1) may have been located within the perivitelline space of the eggs, rather than within the egg proper, i.e., the yolk itself. It is not possible to determine this within the limits of this experimental design. However, in light of the data obtained from the *in vitro* lysozyme experiment (Table 3), and from the spiked egg content experiment, it seems that *F. psychrophilus* can survive within salmonid egg contents.

Surface contamination due to *F. psychrophilus* within the water is a serious concern for the hatchery in question, and this may well be the case for other hatcheries. This hatchery has a fish-free water source. However, amphibians, insects, snails, and possibly other animals may be reservoirs of infection, releasing *F. psychrophilus* into the water. Additionally, *F. psychrophilus* from the water source may be different from the strains found in ovarian fluid, within eggs, or infecting small fish. Tests to clarify these uncertainties are in progress. It also seems likely that the pathogen is transmitted through female ovarian fluid and therefore surface disinfection is critical. It seems likely that there are multiple routes of infection due to *F. psychrophilus* in juvenile salmonids and vertical transmission may be included in these routes. Further work needs to be done to examine the efficacy of broodstock injection with antibiotics.

References

Barker, GA, Smith, SN, & Bromage, NR (1991). Commensal bacterial and their possible relationship to the mortality of incubating salmonid eggs. J.Fish Dis. 14:199-210

Evelyn, TPT, Prosperi-Porta, L, & Ketcheson, JE (1984). The salmonid egg as a vector for the kidney disease bacterium, *Renibacterium salmoninarum*. In: Fish Diseases, 4th COPRAQ Session, Cadiz, Spain, ACUIGRUP, Madrid. pp.111-117

Grinde, B (1989). Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. J. Fish Dis. 12:95-104

Yousif, AN, Albright, LJ, & Evelyn, TPT (1994). In vitro evidence for the antibacterial role of lysozyme in salmonid eggs. Dis.aquat.Org. 19:15-19

SOME TERATOGENIC AND PATHOLOGIC IMPACT ON FISH AND EMBRYOS IN LAKE MARIUT

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Abstract

The successive changes in environmental and ecological conditions of Lake Mariut resulted into the deterioration of fish embryos and larvae of ***Oreochromis species***. Considerable morphological abnormalities such as the bending of the vertebral column, head and yolk sac deformation, delay of growth in certain organs and the whole body beside the deviation of the body parts from normal ratios were diagnosed during the developmental criteria. The high mortality also indicates the threat of the most common,, adaptable fish species in Egypt.

Introduction

Lake Mariut contributed a good part of the Egyptian inland fisheries. The main fish inhabiting the lake comprises ***Tilapia (Oreochromis spp.)*** which constitute about 88.5% of the total fish catch. The teratogenic and abnormal features observed together with the pathologic symptoms mentioned in this paper have been diagnosed through the study of the embryological characteristics of such species collected from their mother brooders inhabited in lake Mariut.

The importance of this study is derived from the sensitivity and susceptibility of the embryos to the environmental factors and conditions that may reflect on the fish catch of one of the most economically important fish in Egypt.

Materials and methods

Fish samples were collected from fishermen's catches from different locations in lake Mariut during the period from April till September for three successive years and then transferred to the laboratories. The two species under consideration; ***Oreochromis niloticus*** and ***Oreochromis aureus*** are mouth brooders which show a high degree of parental care. The mouth incubation period extends from the time of egg fertilization till complete absorption of yolk sac. The embryos and larvae are obtained from the mouth of the mother brooders.

External an morphological features of some embryos collected from the lake were observed and then compared with the same chronological embryonic and larval stages of the same species developed by using the "induced spawning method" and "artificial insemination method" (Ghabrial,1990) under laboratory conditions with high water quality.

The observations were made using the stereomicroscope WILD. MP 50, (magnification 70 -310x) equipped with a substage light source which is directed through the embryos and larvae (transillumination).

Observations

The examined embryos after six days from fertilization (Fig. 1) showed a well deformed head with a length 0.9 mm. and height 1.1 mm., the deformed yolk sac (1.4 x 1.1 mm.) showed a deviation from the normal ratios which are 2.1 x 1.9 mm. at the same stage under high water quality (Lab.) conditions. The structure and morphological characteristics are similar to the well formed fry with its fully formed eye and operculum but less developed alimentary canal.

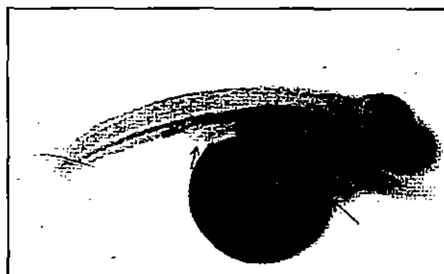

Figure 1. Less developed alimentary canal and deformed yolk sac

Figure 2 is a fully grown larval stage three days after complete absorption of yolk sac showing a short tail region and a clear deformation of the vertebral column at myotome number 5 & 6 of the tail region. The dimensional ratios of the head region were length 1.8 mm. and 1.5 mm. height. indicated a clear deformation from the normal ratios which are 2.2 mm. x 2.1 mm. at the same stage of development.

The number of the anal fin rays were 11+ one spine while the dorsal were 20 fin rays + 4 spines; clearly lesser than the normal numbers which are 15 (anal) and 22 (dorsal fin rays) at the same stage under laboratory conditions.

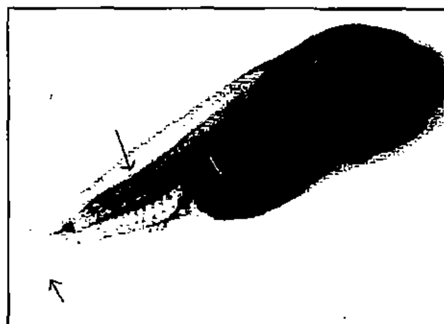

Figure 2. Short tail region and deformation of vertebral column.

The mortality of the fish batch from which those deformed fry were taken was considerable since the original number of eggs was 961 and the surviving fry was about 346.

The fish fry Figure 3, (a & b) show successive developmental stages of the tail region during two successive days showed underdeveloped individuals with clear microcephaly. Ossification of the caudal fin rays was highly affected since the rays became stunted and as if attaining a phalangeal pattern of segmentation, which is quite different from the normal lepidotrichia. Some embryos which were collected from the heavily polluted areas of the lake showed

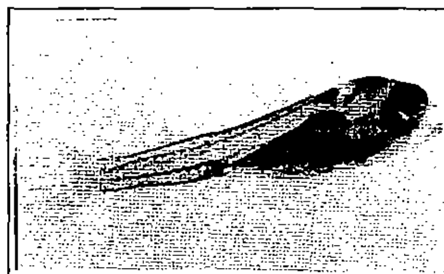

Figure 3a. Ossification of the caudal fin rays.

clear signs of bacterial and fungal infections. Since Tilapia (***Oreochromis spp.***) are present in water with a high organic load due to agricultural, sewage run-off and factories effluent discharged.

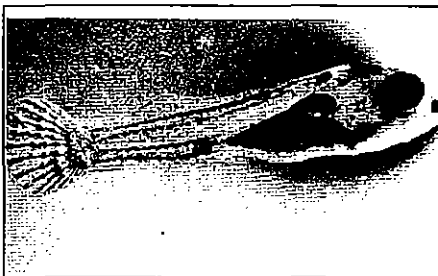

Figure 3b. Stunted rays of the caudal fin showing a phalangeal pattern.

The normal embryonic fin fold of the tail bud and in the zones of the unpaired fins i.e. dorsal and ventral fins show an advanced differentiation reaching a phase where the dorsal fin extends along the entire length and the ventral one almost reaches the anus (age ± 140 h.) after fertilization. At a later stage (± 180 hours) post fertilization, a separation of the dorsal and anal fin folds from the caudal fin is clearly defined at this stage under high water quality conditions (laboratory) (Fig. 4).

Figure 4. Normal dorsal and anal fin rays of a full grown larva stage.

The bacterial and fungal infection which were found in some samples collected from the lake at the same stage of development started as white spots on the dorsal and ventral fins, then increased on the continuous fin fold to show successive deterioration along the surrounding fins (Fig. 5 & 6). The fish fry collected from the same location and kept in the same water of the lake under laboratory conditions for morphological diagnosis at later stages, were found to have a body with high degree of infection with a degenerated tail region and decrease of movement activity and ability of swimming due to loss of appetite.

Figure 5. White spots shown on the dorsal and ventral fins.

Figure 6. Successive deterioration along the fins.

Discussion

The genus ***Tilapia***, which consists of about many species and to which ***Oreochromis spp***. are belonging to, is very economically important. Beside its tolerance to different environmental

conditions and other characteristics such as their hardness, ease of breeding, diversified food habits and fast growth.

The well developed embryonic vascular system proved to have an adaptation that suits the adverse conditions of low oxygen concentration (Ghabrial , 1990), since this species under investigation can survive in habitats of poorly oxygenated areas.

In spite of the tolerance of our species to different environmental conditions, there is nevertheless a wide range of teratogenic problems may occur. The spinal malformations are not uncommon in small numbers in any intensively reared species but in Tilapia (***Oreochromis***), a particular form of spinal deformity, has been mentioned by Roberts and Sommerville (1982). There were "Saddle back"; "spinal deformities" and "dorsal fin anomaly" and they found that such fish is less resistant to diseases such as Sparolegnia fungus infection.

During their waterborne infection tests with infection pancreatic Necrosis virus (IPNV), Ahne W. et al (1989) found that the main symptoms on infected rainbow trout fry were: curvilinear bodies and darkening of caudal body, as well as distinct cranial odema. This indicates that the spinal deformities and the macrocephaly of our fry under consideration may be due to some viral infections from the water of lake Mariut.

The dimensional deviation of some fish larvae from normal ratios is a phenomenon mentioned also by Roberts and Sommerville (1982) on some ***Tilapia*** species, where it was described as "stumped body" in the mature individuals since the body was compressed anterioposteriorly. The incidence of such deformities and abnormalities in the polluted areas in lake Mariut reflects the effect of pollution on fish fry of ***Tilapia*** (***Oreochromis***) which is considered as a highly resistant species.

The high mortality in certain clutches of eggs and larvae is also associated with dwarfish in some individuals. Only 346 fish larvae survived out of about 961 eggs produced from ***O. niloticus*** with 64% loss. Some of which had dwarf larvae if compared with the normal parameters of the same species at the same age and same conditions but different water quality. This dwarfish was also found in the mature ***Sarothorodon mossambicus*** (***Tilapia*** substrate spawner) in lake sibaya (Bruton and Allonson 1974). Iles (1973) emphasized that dwarfism represents an adaptive mechanism involving reproductive and growth characteristics which enable ***Tilapia*** population to withstand high mortality.

Stunting in genus ***Tilapia*** was mentioned by Iles (1973) but the phalangeal pattern of segmentation which was quite different from normal lepidotrichia, was so far, not mentioned. This severe ossification of the fin rays of some individuals in the present study might be due to pollution or to some interaction between certain elements in water and calcium. More work has to be done to identify it physiologically and analytically.

The bacterial and fungal infection of some embryos collected from the heavily polluted areas of the lake at 25°C if maintained at the same temperature, but under laboratory conditions, the infection was severe covering almost the body.

The pathologic bacteria ***myxobacteria*** was described by Roberts and Sommerville (1982) but are usually associated with low water temperature. Avault et al (1968) stress the importance of maintaining over wintering temperature of at least, $\pm$ 4°C for this reason. Therefore, we must point out that the pathogenic infection found during our study might be a different strain other than ***myxobacteria***.

In conclusion, the environmental conditions in the Lake have to be improved, since it is considered a supply for fish fry to many fish-cultures in some near Governerates, to avoid such impact on the embryos and the high mortality associated with these infections.

References

Ahne W., R.K.Kelly and H.J. Schloteldt (1989). Factors affecting the transmission and out break of Infections Pancreatic Necrosis (IPN). "Fish Health protection strategies". Contribution to the Canadian-German Cooperation Program. Edited by Kurt Lillelund & Harald Rosenthal. Hamburg/ Bonn April 1989 (P. 17-67).

Avault, J.W., E.W. Shell and R.O. Smitherman. 1968. Procedures for overwinter ***Tilapia***. FAO Fish. Rep. 44: 343-345.

Bruton, M.N. & Allanson, B.R. (1974). The growth of ***Tilapia mossambica*** Peters (Pisces: Cichlidae) in Lake Sibaya, South Africa. J. Fish Biol. 6: 701-715.

Ghabrial, S. G. (1990): " Induced Spawning and developmental criteria of two ***Oreochromis spp.*** and their hybrid" Msc. Thesis submitted to the Faculty of Science, Alexandria University.101p.p.

Iles, T. D. (1973): Dwarfing or stunting in the genus ***Tilapia*** (Cichlidae); a possible unique recruitment mechanism. Rapp. P -v Reum. Int. Explore. Mer 164 : 247 - 254.

Roberts, R. J. & C. Sommerville (1982). 247- 263 In R. SV. Pullin and R. H. Lowe - Mc Connell (eds. - The biology and culture of tilapias. ICLARM Conference proceedings 7, 432 P. ICLARM, Manila, Philippines.

VIABILITY OF MILKFISH EGGS AND LARVAE AFTER SIMULATED AND ACTUAL TRANSPORT

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Abstract

The viability of milkfish eggs and larvae after simulated and actual transport was investigated. Naturally-spawned milkfish eggs were collected and subjected to simulated or actual transport at early cleavage stage (stage 1), blastula (stage 2), gastrula (stage 3), "eyed" (stage 4), or newly-hatched larvae (stage 5). Replicate samples in aerated plastic jars served as controls. Mean hatching and survival rates and the percentage of newly-hatched larvae were significantly affected by the modes of transport and by the stage of embryonic development at transport. Eggs transported at the 'eyed' stage had higher viability compared to those transported at cleavage, blastula, or gastrula stages. There was no significant difference in the mean survival rate of the larvae after 26 days of rearing. However, the percentage of 45 day old larvae with apparent morphological abnormalities was lower in groups transported at stages 4 and 5. These observations indicate that milkfish eggs should be handled and transported during the late embryonic stages to minimize mortalities and the incidence of abnormalities in larvae.

Introduction

Milkfish, *Chanos chanos* Forsskal, is one of the important cultured fish in Southeast Asia. Since milkfish was reported to spontaneously spawn in cages (Marte and Lacanilao, 1986), ponds (Lin, 1985), and tanks (Pjorono, 1988; Emata and Marte., 1993), hatchery-bred fry were made available to fish farmers. In the Philippines, there are anecdotal reports about the high incidence of osteological abnormalities in hatchery-reared milkfish fry. Hilomen-Garcia (in press) recently characterized the morphological abnormalities in hatchery produced milkfish juveniles.

Available literature suggest that osteological abnormalities in hatchery-reared fish may be induced during embryonic and post-embryonic stages by some environmental factors(Houde, 1973; Barahona-Fernandes, 1982; Longwell et al., 1992). Deformities in the embryos and skeleton in some fish has been attributed to aquatic pollution such as in winter flounder (Perry et al., 1991) and perch (Lindesjoo et al., 1994), to mechanical stress during routine hatchery operations (Daoulas et al., 1991; Kitajima et al., 1994), or to nutritional defficiencies (Kanazawa, 1985).

In the Philippines, naturally spawned eggs are presently transported from the broodstock floating net cages to land based hatcheries for rearing to fry or fingerling stage. Fertilized eggs are collected within an hour after spawning and eggs are routinely packed in oxygenated plastic bags during blastula to neurula stage for transport (Garcia and Toledo, 1988; Emata and Marte, 1993).

Milkfish fish farmers often complain about the large numbers of malformed marketable size milkfish they produce from hatchery seeds.

This study was conducted to investigate the viability of milkfish eggs and larvae after simulated and actual transport. Viability was determined based on survival after transport, hatching rate, occurrence of lordosis in newly-hatched larvae, survival of larvae after metamorphosis, and the prevalence of gross morphological abnormalities in fry. Based on the results, recommendations to improve viability of eggs after handling and transport were made.

Materials and Methods

Egg Collection and Handling

Spontaneously spawned milkfish eggs from a floating net cage were collected within 30 min to 1 hr after spawning. Broodstock conditions were as described by Emata and Marte (1993). Spawned eggs were collected following Garcia et al. (1988). Collected eggs were temporarily stocked in a 2x2 1.5 m hapa net cage prior to their use. Moderate aertation was provided to prevent the eggs from clumping.

Simulated and Actual Transport

Viability of milkfish eggs and larvae after handling and transport at different developmental stages was examined. Milkfish eggs were subjected to simulated or actual transport conditions at early cleavage (stage 1), blastula (stage 2), neurula (stage 3), "eyed" stage (stage 4), or at hatching (stage 5).

Eggs or larvae were transferred from the hapa net cage into three replicate plastic bags (10x20 cm) containing 100 ml of ambient sea water at a stocking density of 1,000 eggs/l. Plastic bags were inflated with oxygen at a ratio of 1:2 (water:oxygen). To simulate actual transport conditions, packed plastic bags were placed in a tray secured on top of a laboratory shaker and shaken at 50 rpm for 2 hours (treatment A).

Eggs or larvae were transported to SEAFDEC's Tigbauan hatchery which is about 2-3 hours travel by land and sea (treatment B) following routine procedures as described by Gapasin and Marte (1990). Briefly, eggs were packed in double lined plastic bags containing 5 liters of ambient sea water at a density of 10,000 eggs per liter. For each stage, three replicate samples were separately incubated in a moderately aerated 1 liter plastic jar at a density of 200-300 pcs/l and served as controls (treatment C).

Larval Rearing

To examine the effects of transport at different embryonic stages on the incidence of abnormalities in hatchery bred milkfish fingerlings, larvae hatched from eggs at actual transport conditions were reared separately in three replicate 250-l conical tanks following Gapasin and Marte (1990), with some modifications. Initial stocking density was 30 ind/l. Larvae were initially fed lipid-enriched rotifer at a density of 15 ind/ml from Day 2 to Day 15. Lipid-enriched artemia metanauplii was given to satiation from Day 15 to Day 25. Total harvest was done on Day 26. Samples (25-30) were taken from each tank for individual growth measurements. The remaining larvae from each stage were pooled and further reared to Day 45 in a 1 ton fiberglass tank. The larvae were totally harvested on Day 46 and preserved in 5% buffered formalin for examination of morphological abnormalities.

Determination of Viability

Twenty ml aliquot samples of transport water (200-300 pcs/sample) were taken from each replicate bags immediately after simulated or actual transport and transferred to 1 liter plastic jars containing ambient sea water and provided with moderate aeration. To determine the survival rate after transport, aeration was stopped 3 hours later for at least 5 minutes and dead eggs were pippeted out individually. Dead eggs of milkfish are opaque and sink at the bottom (Juario et al., 1984). Hatching rate was examined by counting the total number of unhatched eggs and hatched larvae in a jar. The number of normal, dead and moribund larvae, and larvae with lordosis were noted. The prevalance of external morphological abnormalities were also investigated in Day 46 old fry by randomly taking three teaspoonful of aliquot samples from preserved specimens.

Statistical Analysis

A 3x5 factorial in a completely randomized design was used in the experiment. Data were analyzed by SAS statistical program (1988). Means were arc transformed to correct the unequal distribution of sample sizes. Three-way or one-way ANOVA was used to determine the effects of types of transport and stages of embryonic development on the viability of eggs followed by DMRT to compare significant differences between means at P>0.01.

Results

Figures 1, 2, and 3 show the viability of milkfish eggs or larvae after simulated or actual transport at different embryonic stages. There was a significant interaction between the modes of transport and the stage of embryonic development during transport. Mean survival rates, hatching rates, and the percentage of larvae with lordosis at different stages varied significantly with the modes of transport used. Viability of eggs in terms of survival after transport, hatching rate, and prevalence of newly hatched larvae with lordosis in the control group was higher than those subjected to simulated or actual transport. No significant difference in the mean survival and hatching rates of eggs was observed between stages 1-4 in the control group. Milkfish eggs transported at stage 4 had significantly higher survival and hatching rates than stages 1, 2, and 3 in both simulated and actual transport conditions. Survival after transport was relatively lower in newly-hatched larvae (Stage 5) than in eggs (Stages 1, 2, 3 and 4). Newly-hatched larvae with lordosis varied significantly from 10.3% in the 'eyed' stage of control (treatment C) to 60.1% in those transported at gastrula (treatment A).

The percentage of dead or moribund larvae among lordotic newly-hatched larvae progressively decreased in eggs subjected to actual transport (Fig. 4) at stage 1 (65.6%) to stage 4 (30.9%). Gross morphological abnormalities in 45 day old fry were significantly lower in groups transported at stage 4 (19.9%) and stage 5 (9.7%) than other stages tested (Fig. 5).

There was no significant difference in the mean survival rates (6.7-17.2%) of larvae transported at stages 1-5 after 26 days of rearing. However, mean total length of 26 day old larvae was significantly higher in those transported at stages 4 (12.19 mm) and 5 (12.73mm) than stages 1 (11.39 mm), 2 (11.36 mm), and 3 (11.57 mm).

Ambient air temperature and salinity during the handling and transport tests varied from 28.2 C to 29.8 C and 32 ppt, respectively. Dissolved oxygen of water in all plastic bags was above 5.6 ppm after transport.

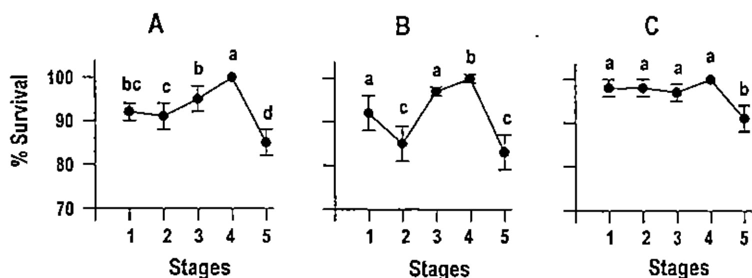

Figure 1. Percentage survival of milkfish eggs or larvae 3 hrs after actual (A) or simulated transport (B). C, control; stage 1, early cleavage; stage 2, blastula; stage 3, gastrula; stage 4, 'eyed'; and stage 5, newly-hatched larvae. Points are means ± standard deviation of 3 replicates. For each figure, means with the same superscript are not significantly different (P>0.01).

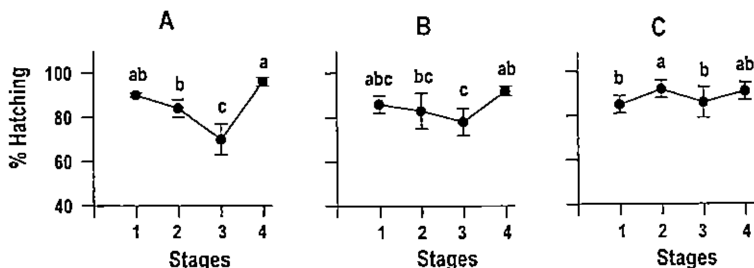

Figure 2. Percentage hatching of milkfish eggs after actual (A) or simulated (B) transport. C, control; stage1, early cleavage; stage 2, blastula; stage 3, gastrula,; and stage 4; 'eyed'. Points are means ± standard deviation of 3 replicates. For each figure, points with the same superscript are not signicantly different (P>0.01).

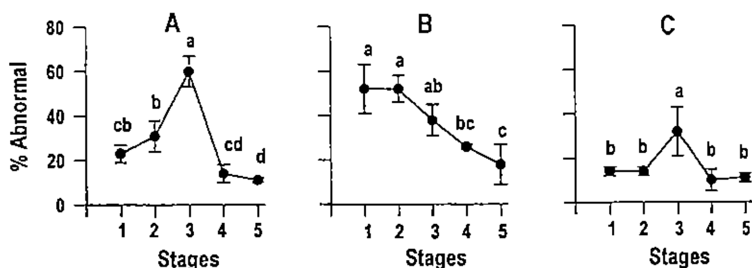

Figure 3. Percentage of newly-hatched larvae with lordosis after exposure of milkfish eggs or larvae to actual (A) or simulated (B) transport. C, control; stage 1, early cleavage; stage 2, blastula; stage 3, gastrula; stage 4, 'eyed'; and stage 5, newly-hatched larvae. Points are means ± standard deviation of 3 replicates. Means with the same superscript are not significantly different (P>0.01).

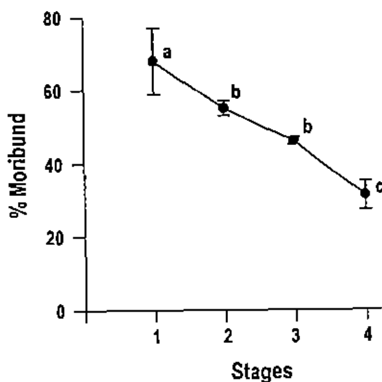

Figure 4. Percentage of moribund and dead larvae among lordotic newly-hatched milkfish larvae after actual transport of eggs. Stage 1, early cleavage; stage 2, blastula, stage 3, gastrula; and stage 4, 'eyed'. Points are means ± standard deviation of 3 replicates. Means with the same superscript are not significantly different (P>0.01).

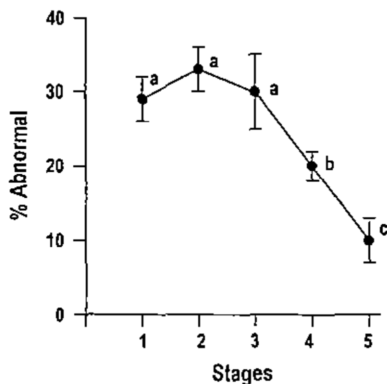

Figure 5. Percentage of 46 day old larvae with gross morphological abnormalities in milkfish eggs or larvae after actual transport. Stage 1, early cleavage; stage 2, blastula; stage 3, gastrula; stage 4, 'eyed'; and stage 4, newly-hatched larvae. Points are means ± standard deviation of 3 replicates. Means with the same superscript are not significanly different (P>0.01).

Discussion

This study demonstrates that handling and transport at different stages of embryonic development affect the viability of milkfish eggs or larvae. Mean survival and hatching rates as well as the percentage of newly-hatched larvae with lordosis were significantly affected by the modes of transport and the stages of development during transport.

Viability was significanly higher in milkfish eggs transported at late embryonic development than those at earlier stages (Figs. 1, 2 and 3). Our present results agree well with those reported in other fish species (Jensen and Alderdice, 1983; von Westernhagen, 1988). Increasing levels of sensisitivity to mechanical stress from fertilization to the gastulation stage was observed in coho salmon by Jensen and Alderdice (1983). Sensitivity declined at the beginning of completion of epiboly until the early eyed stage. Early embryonic stages (before gastrulation) of a variety of freshwater eggs are more vulnerable to aquatic pollutants than those that have completed epiboly (von Westernhagen, 1988). In this study, mean survival and hatching rates were significantly lower in eggs transported at the cleavage, blastula, and gastrula stages compared to those at the eyed stage. A significantly higher incidence of newly-hatched larvae with lordosis and higher number of dead or moribund hatchlings was similarly observed in stages 1, 2, and 3 than stages 4 and 5.

Lordosis in newly-hatched larvae were also observed in the control group. Fertilized eggs in this study were collected from the floating net cage during two to four cell stages which were considered to be sensitive to mechnanical stress in coho salmon (Jensen and Alderdice, 1983). It is not clear in our present results whether the presence of abberant newly-hatched larvae in the control groups was caused by mechanical stress during egg collection or by other factors.

In most cases, survival of milkfish eggs was significantly higher than newly-hatched larvae. Fine mesh scoop nets were used in this study during handling and transfer of eggs or larvae. While newly-hatched larvae were left naked during abrupt dehydration during handling and packing, milkfish embryos were protected with an outside covering (chorion) and a small perivitelline membrane that covers the embryo (Juario et al., 1984). Fertilized milkfish eggs exposed in air for about 3 min in a wet fine scoop net were observed to develop normally until hatching (JD Toledo, personal observations).

Although no significant difference in survival was observed in larvae transported at different embryonic stages after 26 days of rearing, total length of the larvae transported at stages 1, 2, and 3 was significantly shorter than those transported at stages 4 and 5. The incidence of gross morphological abnormalities in Day 46 larvae was also significantly higher in groups transported before the eyed stage (Fig. 5). Almost all the abnormalities were observed in the head region with high prevalence in the operculum. Hilomen-Garcia (in press) similarly observed that milkfish with opercular and branchiostegal abnormalities had slow growth and had high mortality rate after handling and transfer. Cell damage due to transport stress may have been lethal to embryos at earlier stages as reflected by the significantly high incidence of dead and moribund larvae (Fig. 4). Sublethal damage incurred at these stages may have been repaired as the larvae developed and was discernable only at the juvenile stage.

Based on the results, fertilized milkfish eggs should be transported at least past the gastrulation phase, preferably at more advanced developmental stages to minimize mortalities and lower the incidence of abnormal larvae in the hatchery. At ambient temperature and salinity, it takes about 14-16 hours after fertilization to reach the C-shaped embryo with formed optic vesicles which was shown in this study to be the best stage for transport resulting in highest viability. Fertilized milkfish eggs are sometimes required by hatcheries several hours away and eggs transported at late embryonic stage may hatch along the way if transport water temperature is not lowered. In this case, newly-hatched larvae should be transported at far distances instead of eggs.

Acknowledgements

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References

Barahona-Fernandez, MH 1982 Body deformation in hatchery reared European sea bass Dicentraschus labrax (L). Types, prevalence and effect on fish survival. J. Fish Biol., 21:239-249.

Daoulas, Ch, AN Economou, and I Batavas 1991 Osteological abnormalities in laboratory reared sea bass (Dicentrarchus labrax) fingerlings. Aquaculture, 97:169-180.

Emata, AC and CL Marte 1993 Broodstock management and egg production of milkfish, *Chanos chanos* Forsskal. Aquacult. Fish. Man., 24:381-388.

Gapasin, RSJ and CL Marte 1990 Milkfish hatchery operations. Aquaculture Extension Manual No. 17, 24 pp. Tigbauan, Iloilo, Philippines. Aquaculture Department, Southeast Asian Fisheries Development Center.

Garcia, LMB, CL Marte, and VS Travina 1988 A collecting gear for naturally-spawned milkfish (*Chanos chanos* Forsskal) eggs in circular net cages. Aquaculture, 68:83-86.

Garcia, LMB and J.D Toledo 1988 Critical factors influencing survival and hatching of milkfish (Chanos chanos Forsskal) eggs during simulated transport. Aquaculture, 72:85-93.

Hilomen-Garcia, GV In Press Morphological abnormalities in hatchery-bred milkfish (*Chanos chanos* Forsskal) fry and juveniles. Aquaculture, 0:00.

Houde ED 1973 Some recent advances and unsolve problems in the culture of marine fish larvae. Proc. Wld. Maricult. Soc., 3:83-103.

Jensen, JOT and DF Alderdice 1983 Changes in the mechanical shock sensitivity of coho salmon (*Oncorhynchus kisutch*) eggs during incubation. Aquaculture, 32:303-312.

Juario, JV., MN Duray, JF Nacario, and JME Almendras 1984 Induced breeding and larval rearing experiments with milkfish *Chanos chanos* (Forsskal) in the Philippines. Aquaculture, 36:61-70.

Kanazawa, A 1985 Essential fatty acid and lipid requirement of fish. In: CB Cowey, AM Mackie and JG Bell (Editors), Nutrition and Feeding in Fish. Academic Press, London. pp.281-298.

Kitajima, C, T Watanabe, Y Tsuhima, and S Fujita 1994 Lordotic deformation and abnormal development of swim bladders in some hatchery bred marine physoclitus fish in Japan. J. World Aquacult. Soc., 25:64-77.

Longwell AC., S Chang, A Hebert, JB Hughes, and D Perry. 1992. Poluution and developmetal abnormalities of Atlantic fishes. Environmen. Biol. Fishes, 35:1-21.

Lin, LT 1985 My experience in artificial propagation of milkfish - Studies on natural spawning of pond-reared broodstock. In: CS Lee and IC Liao (Editors), Reproduction and Culture of Milkfish. Oceanic Institute and Tungkang Marine Laboratory. pp.185-203.

Lindesjoo, E, J Thulin, BE Bengetsson, and U Tjarlund 1994 Abnormalities of a gill cover bone, the operculum, in perch Perca flutavilis from a pulp mill effluent area. Aquat. Toxicol., 28:189-207.

Pjorono, A, NAG Tridjoko, A Poernomo, WE Vanstone, C Lim and T Daulay 1988 Natural spawning and larval rearing of milkfish in captivity in Indonesia. Aquaculture, 74:127-130.

Marte, CL, and F Lacanilao 1986 Spontaneous maturation and spawning of milkfish in floating net cages. Aquaculture, 53: 115-132.

Perry, DM., JB Hughes, AT Hebert. 1991. Sublethal abnormalities in embryos of winter flounder, *Pseudopleurenectes americanus*, from Long Island Sound. Estuaries, 14:306-317.

SAS Inst. Inc. 1988 SAS/STAT Users Guide, Release 6.03 edition, 1028 pp. North Carolina: SAS Inst. Inc., Cary.

von Westernhagen, H 1988 Sublethal effects of pollutants on fish eggs and larvae. In: W.S. Hoar and D.J. Randall (Editors), Fish Physiology, Vol. 11, Part A. Academic Press, New York. pp. 253-330.

DEVELOPING SPAWNING AND INCUBATION TECHNIQUE FOR MIGRATORY MAHSEER IN THE HIMALAYAN RIVERS OF NEPAL

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Abstract

Number of migratory mahseer *Tor putitora* is declining due to over-fishing, environmental pollution and habitat modification due to power dams. For conservation and management of this endangered game fish require special incubation techniques. Technique has been developed for spawning and rearing in natural and artificial substrata and introduction hatchery bred fries into depleted natural population. Fertilized eggs of mahseer up to fry stage has been studied in intergravel water. The field and laboratory results show that there is possibility of augmenting the mahseer population of the migratory mahseer by artificial breeding, releasing and replenishing the depleted stock. The paper also highlights need of developing novel type spawning channel or incubation and incubating facilities for increasing mahseer fry resources for faster propagation.

Introduction

The mahseer (*Tor putitora*) are large endangered cyprinid occurring in the snowfed Himalayan waters of Nepal, Shrestha (1990). The scientific literature of consists only a few fairly rudimentary remarks on natural and artificial breeding and incubation of mahseer eggs. A preliminary study on migration and spawning was furnished by Shrestha (1994). The literature on spawning and incubation of Salmonid eggs is voluminous (Baily and Taylor, 1974 and Bam and Simpson, 1976). But the published information on concerning spawning and incubation of mahseer eggs is scanty because mahseer is vary difficult to breed in captivity and yet no reliable incubation techniques have been developed for faster propagation of this species. In the present paper an attempt is made study incubation success of the mahseer eggs by using different incubating devices in the mountain stream condition of Nepal.

Material and Methods

In the past three years, I have tried to locate natural spawning sites of mahseer around feeder streams of Trisuli (feeder stream creeks such as Tadi, Sindurae, Khahare etc.). However, I found only one spawning site near Gadkhar fish farm at Chokedovan (Fig. 1) which was found to be utilized for spawning year after year. Field observation was carried out from 1993 - 1995 at this site and period and duration of spawning, size and number of individuals in each spawner group were recorded. The water quality and hydro-biological parameters of the study area were

noted. The migrants were also captured to determine their physical conditions and to study spawning behavior in captivity. The propagation of the mahseer is detailed in my previous studies (Shrestha 1990, 1992).

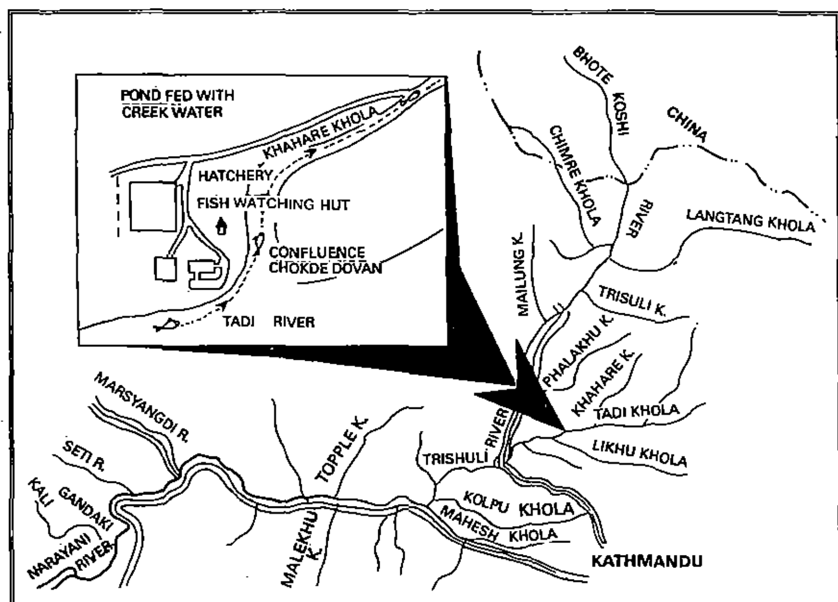

Figure 1. Showing Study Area

Adult mahseers were also trapped from the creeks at night by means of gill nets. They were put in a large cloth tank or happa (size 4X3X2m) for acclimatization. The happas were suspended in the flowing creek water. Inside happa male and female fishes were put in a ratio 3:1 and were administered with carp pituitary extract injection (0.4gm/body weight of fish). Fishes were fed twice daily with rice bran, oil cake etc. Stripped eggs from breeders were fertilized with wet and dry methods and reared in a series of incubators by putting them safely in semi-natural incubating channel.

Observation

Incubation channels were constructed in creek nearby the Gadkhar hatchery where fertilized fish eggs are artificially buried rather allowing the fish to deposit eggs naturally. In the incubation channels fish eggs can be stocked in higher densities. Densities (2000 eyed eggs/ft^2) of surface area yield high survival in fry stage. A variety of incubators both wooden or plastic incubators of different sizes were used to incubate mahseer eggs in the incubation channel. Dead eggs were removed or siphoned. The advanced fish fry after hatching drops from the egg incubator and work their way into gravel substrate. Preferred flow mahseers are considered to be 1.0-1.5 cfs. The riverside incubation channel is effective for golden mahseer eggs.

The experimental incubation channels do need sophisticated hatchery operation, simple facilities available near water mill (Ghatta) was used to build incubation facilities 1) Requires less space 2) increases survival of eyed egg and fry over natural production because of

controlled flow and clear water from spawning creek 3) less capital cost. Transfer of Salmonid incubation technology developed in USA found to be useful on mahseer living in ecological condition of mountain rivers of Nepal.

Use of Artificial Incubators for Incubation

A typical incubation channel constructed in Gadkhar at embankment of creek (Khahare Khola) is of 6 ft wide, 50 ft long and 12 inches deep. The gravel size varied 0.5 to 2 inch in size. Fertilized eggs were placed in turf or plastic incubators with small orifice at top for allowing rapid dispersion of swim up fries. The fries development in the gravel substrate and their emergence time was recorded (Table 1, 2 and 3).

The water was supplied to the channel from Gadkhar creek. The silt free spring water was also tried and had been very much successful and insured higher survival than natural water of the river. For incubation, besides plastic incubators and metal or wooden turf incubators having 4 X 4 X 4 ft were used.

An experimental natural spawning channel at the confluence of Tadi river and Khahare Khola (creek) was selected due to optimum gravel bed and water flow condition for spawning and incubation. The gravel size in the creek was 0.5 to 0.6 inches. The gradient of creek was 0.2 to 0.5 percent which promoted good interchange between surface and intergravel water. During the peak spawning season (September) water depth as 1.5 feet was present. To study natural incubation under natural condition freshly fertilized eggs were put into freshly prepared gravel beds with controlled flows of water or regulated flow condition was allowed to run naturally over the spawning assembly of the gravel beds or fertilized egg produced by stripping male and female mahseer is manually buried in an incubation channel. The hatching development and remission of the fry took 240 hrs.

Discussion

On the basis of my field observations in the incubation channel at Gadkhar creek, it was noted that the incubation of mahseer eggs in the creek was always associated with low water level, monsoon rain. Sinha, Jhingran and Ganapati (1974) indicated that no single factor could yet be recognized as the most important for spawning and early development major carp such as mahseer. The present study also suggests many factors as noted to be responsible for the successful spawning, development and incubation are (i) clean and continuous water flow and steady water level, (ii) increasing current velocity. High turbidity (low transparency), (iii) optimum water surface temperature (28 to 30 °C), (iv) high level of dissolved oxygen and conductivity (8 to 10 ppm), (v) slight acidic nature of water, (vi) presence of fine gravel with intergravel flow, oxygen retaining red and particles rich in ferro-magnetic or volcanic ashes protect embryo from infection of parasites. The interaction of all these factors provides unique hydrological conditions at the semi-natural incubation ground which might provide rheotactic effects for effective incubation.

In fish farm of Nepal, mahseer sac fry is prone to infection of molds *Saprolegnia* because sacfry undergo long periods of latency extending from 48 to 92 hrs. In future more effect incubation system suited in the ecological condition of mountain stream to be developed.

Mean incubation periods of fry in the Gadkahar hatchery were estimated at 6 to 8 days. But incubation periods in creek were estimated at 8 to 12 days depending on the temperature of creek water. In creek developmental rate is slower than in hatchery due to lower temperature and intergravel flow.

Table 1
Gravel Incubators Mahseer Eggs Subjected to Incubation at Different Temperature and Velocity

Field Sites	No. of Gravel Incubators	Incubating Temperature °C (Mean)	No. of eggs Incubated	Gravel size (mm) (mean)	Average current velocity m/sec (Mean)	Dissolved Oxygen (ppm)	Time taken for hatching	Time taken for emergence as fry (hrs)	No. of eggs hatched (Mean)
Mouth of creek	5	30	21500	50	1.2	9	48	240	21423
Middle part of creek	5	28	21500	28	0.8	7	60	232	21390
Confluence site of creek and stream	5	22	21500	12	0.0	10	72	215	21218

Table 2
Comparative Chart Showing Incubation Success in Different Incubators

Type of Incubators and Dimension	Gravel Size in Inch	No. of Egg Incubated	No. of Egg Hatched as Sacfry	Hatching Time (hrs)	Hatching Temperature (°C)	Time taken for emergence as fry (hrs)	Dissolved Oxygen (ppm)	Current velocity m/sec
Wooden Incubator	0.5 to 2	2000	1896	72	25	216	10	0.5
Plastic Incubator	0.5 to 2	2000	1843	70	28	210	10	0.5
Turf Incubator	0.5 to 2	2000	1940	72	30	192	10	0.5

Table 3 :- Water Quality of Mahseer Incubation Channel at Gadkhar Creek

Temperature	30 °C
Color	Brown
Visibility (m)	0.36
Compensation depth	0.82
pH	7.6
Oxygen	10.5
Dissolved solids	158.5
Hardness	47.0
Specific conductance	287.0
Chloride	18.5
Sulphate	14.5
Calcium	23.0
Magnesium	2.8
Iron	0.11

References

Bailey, JE and Taylor, SG 1974. Salmon fry production in a gravel incubator hatchery. Auk Creek, Alaska, 1971-1972. NOAA. Tech. Memo. NMFS, ABFL. 3. 13 p.

Bams, RA and Simpson, KS 1976. Substrate incubator workshop. Report on current state of Art . Fisheries and Marine Service. Environment Canada.

Shrestha, TK 1986. Ecology and behavior of Mahseer *Tor putitora* Hamilton in the Himalayan Water of Nepal. In J.L. Maclean, L.B. Dizon and L.V. Hosillos (eds.) The First Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines. 689-692 p.

Shrestha, TK 1990. Rare Fishes of Himalayan Waters of Nepal. J. of Fish Biology 37 (Supplement A) Academic Press, London. 213-219 p.

Shrestha, TK 1992. Propagation of Mahseer in the Himalayan Waters of Nepal. In: National Research Council. 1992. Aquaculture and Schistosomiasis: Proceedings of a network meeting held in Manila, Philippines, August 6-10, 1991. National Academy Press. Washington, D.C. 61-78 p.

Shrestha, TK 1994. Migration and Spawning of Golden Mahseer in Himalayan Waters of Nepal. J. Freshwater Biol. 6(1): 71-77.

Sinha, VRP, Jhingran, VG and Ganapati, SV 1974. A review on spawning of the Indian major carps. Arch. Hydrobiol. 73: 518-536.

Egg Quality

PARENTAL SIZE AND PERCEIVED BROOD VALUE:

ARE ALL EGGS CREATED EQUAL?

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Abstract

We report a negative correlation relating maternal weight and magnitude of defense within convict cichlids (Cichlasoma nigrofasciatum). These results illustrate that cichlids maximize return on reproductive investment by evaluating the relative returns that can be reaped from present and expected future broods.

Introduction

In a broad sense, we explored parental investment decisions which comprise an important dimension of life history strategies. Life history theory allows us to predict, test and understand different adaptive aspects of an organism's life cycle (Stearns, 1977). A life history perspective defines the phenotype by demographic characters, such as age, size, number of offspring and growth and reproductive investment. These fitness components influence each other through interrelated trade-offs, arising from functional constraints.

These components vary not only between species, but also between individuals within a species and even within the life cycle of an individual. Demographic characters will change throughout a life history, as will, therefore, the life history strategy at any given point. Indeterminate growth in fish means that size will be a continuously varying factor for an individual, giving rise to a plastic strategy within the life history of a single individual.

We focused on the relationship between present and future investment. Viewing present investment in the context of future reproductive prospects is appropriate, as resource allocation in the present influences not only current reproductive success, but also future reproduction. This is to say expenditure on a current brood has repercussions on the condition of the parent and therefore may compromise ability to invest in future broods (Coleman et al., 1985; Coleman and Gross, 1991).

Maximization of reproductive investment, and for that matter any form of investment, is directly derived from the rate of return on the investment. If an animal maximizes this quantity at each point within its life history, taking into account present conditions and future prospects, on average it will ultimately achieve the highest possible return on its investment (Sargent and Gross, 1985; Sargent and Gross, 1992).

We approached this issue by asking if parents who have different expected future reproductive success would value the same brood size equally. In fish, brood size increases with female size. Therefore, we questioned whether smaller parents value a fixed number of offspring more highly than do larger parents. This is a reasonable prediction, given that if a smaller parent respawned, it would expect a smaller brood than if a larger parent respawned.

Reproductive effort encompasses costs in terms of both energy and risk. We assumed that these efforts rise with perceived brood value. The particular parameter we took as a reflection of perceived value was the magnitude of defense a parent was prepared to expend against a brood predator. Not only does this measure incorporate both energy and risk components, but it is also relatively straightforward to measure through manipulative experiments.

We selected the convict cichlid (Cichlasoma nigrofasciatum) as our study animal, because this fish exhibits extensive parental care in the form of guarding and fanning (Lavery and Keenleyside, 1990; Keenleyside, 1991; Keenleyside et al., 1990). Moreover, pair bonding is not for life in convicts, so there is no complication arising from the value of maintaining a bond between parents.

Methods

We set up sixteen aquaria using females ranging from 4 g to 14 g. Females of various sizes were deliberately chosen to maximize the span of sizes examined. No attempt was made to control for male size because males were removed for the parental defense tests.

Each pair of fish was bred in fifteen gallon aquaria, three sides of which were covered with paper to provide visual isolation. Each aquarium contained two cm of gravel, a plastic plant and a bottomless flower pot measuring 8.8 cm in diameter to serve as a suitable spawning substrate. Each tank also had a heater to maintain the temperature at 28°C which is conducive to breeding. Room lighting was 12L:12D with fifteen minutes of simulated dusk and dawn. The fish were fed daily with frozen brine shrimp and TetraCichlid flakes.

The fish were checked at least once a day for spawning. The day after spawning, recorded as day 2, the pot was removed to count the number of eggs laid on it. A scraper was then used to remove any eggs in excess of one hundred. The weights of both parents were measured on an electronic balance and the standard and total lengths were measured with calipers. The female was returned to the brood, but the male was removed to another aquarium. Testing the investment of only one of the parents avoids complications arising from biparental interactions of convict cichlids (Coleman, 1992).

On day 6, by which point the offspring had typically reached the free-swimming stage, predator encounter experiments were performed. We constructed a predator model from a photographic print of a non-conspecific brood predator (Tilapia mariae) of total length 55.6mm and standard length 45.5mm, corresponding to a female weighing roughly 5g. The photograph was coated in clear epoxy resin and attached to a plexiglass handle.

For each defense test, the model was slowly inserted into the corner of the tank furthest from the fry and held in this position for five seconds. The model was moved near the site of schooling fry, and the stopwatch was started when the model reached this position. Then the

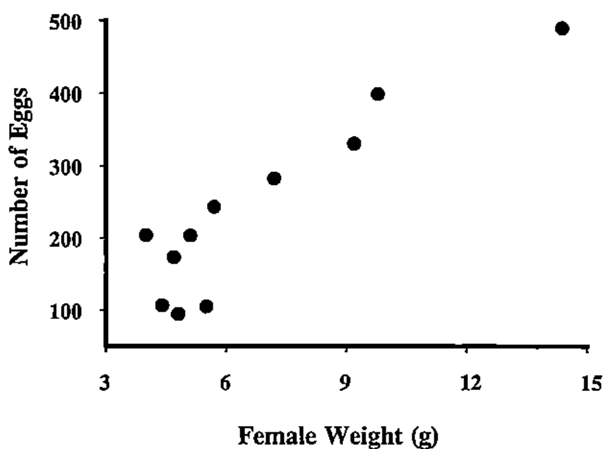

Figure 1. Fecundity versus body size in convict cichlids

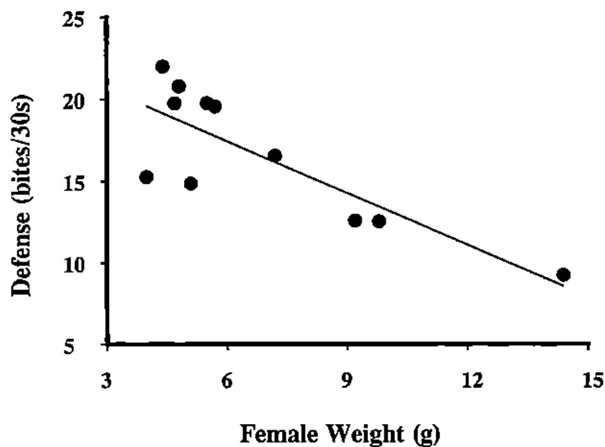

Figure 2. Defense versus body size in convict cichlids

model was moved in a figure-eight pattern, and the number of bites the mother gave to the model within a thirty second period was counted, whereupon the model was withdrawn. After a further thirty seconds, the model was reinserted and the encounter repeated. Over six consecutive days, this entire procedure was performed twice daily, allowing at least thirty minutes between repeats. After the last repeat of the final day, the fry were siphoned out and counted to ensure that the number had not fallen substantially below one hundred. We also weighed the female again and recorded her standard and total lengths. For analysis, we averaged the twenty four test scores obtained for individual females to produce a single defense test score for each female.

Results

We had a total of eleven spawnings from females dispersed over a wide spread of sizes. We found a highly significant positive correlation between number of eggs spawned and the weight of the mother (Fig. 1; $r=0.84$, $df=9$, $p<0.001$). This supports the assumption that fecundity increases with female size.

Magnitude of defense decreased with the size of the female (Fig. 2; $r=0.68$, $df=9$, $p=0.002$).

Discussion

These results are explicable if the mother is indeed acting to maximize the return on her reproductive investment. Optimal allocation is determined by the relative investment returns that can be reaped from present and expected future broods. A larger female can expect to have a larger brood than a smaller female, making a brood of a given size relatively less valuable to the larger female. Consequently, a larger female may be better off conserving expenditure on an existing brood to provide it for subsequent broods by decreasing shareable expenses (sensu Wittenberger, 1981) such as defense against predators.

It is also important to consider that a small fish is likely to become a large fish if it survives. Furthermore, within a single brood cycle, the loss of eggs through predation will be a common occurrence. Consequently, within a lifetime, or even within a brood cycle, parents are continually readjusting their strategies. Consider that in biparental species, such as convicts, there will also be an interplay between the relationship of parent relative to parent in addition to the relationship of parent relative to brood. From an ethological perspective, we can surmise that convicts are able to incorporate dimensions of present conditions in the formation of a life history "decision". A further extension would be to repeat these experiments on a female as it progresses through its life. This should demonstrate that parents are able to change their perception of relative brood value with growth.

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References

Coleman RM and Gross MR (1991) Parental investment theory: the role of past investment. Trends in Evolution and Ecology 6:404-406

Coleman RM, Gross MR and Sargent RC (1985) Parental investment decision rules: a test in bluegill sunfish. Behavioural Ecology and Sociobiology 18:59-66

Keenleyside MHA (1991) Parental care, p. 191-208; in Cichlid Fishes: Behaviour, ecology and evolution. Keenleyside MHA (ed) Chapman and Hall, New York, New York

Keenleyside MHA, Bailey RC and Young VH (1990) Variation in the mating system and associated parental behaviour of captive and free-living Cichlasoma nigrofasciatum (Pisces, Cichlidae). Behaviour 112:202-221

Lavery RJ and Keenleyside MHA (1990) Parental investment of a biparental cichlid fish, Cichlasoma nigrofasciatum, in relation to brood size and past investment. Animal Behaviour 40:1128-1137

Sargent RC and Gross MR (1985) Parental investment decision rules and the Concorde fallacy. Behavioural Ecology and Sociobiology 17:43-45

Sargent RC and Gross MR (1992) Williams' principle: an explanation of parental care in teleost fishes, p. 275-293; in The behaviour of teleost fishes (2nd edn). Pitcher TJ (ed) Croom Helm, London

Stearns SC (1977) The evolution of life history traits. A critique of the theory and a review of the data. Annual Review of Ecology and Systematics 8:145-171

Wittenberger JF (1981) Animal Social Behavior. Duxbury Press, Boston.

EVOLUTION OF EGG SIZE IN NEOTROPICAL CICHLID FISHES

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Abstract

Not all fish eggs are the same. The Cichlidae present a unique opportunity to examine the selection pressures shaping egg size because the family is large, diverse, and found in many ecological niches. The result is dramatic variation in egg size across species. I will describe a series of experiments exploring these selection pressures in Neotropical cichlids, including development time versus temperature, development time versus egg size, egg survivorship versus temperature, and spawning site choice versus temperature. In brief, higher temperatures shorten development time, while larger egg size extends it. And, despite the finding that eggs survive well at high temperatures, parental cichlids choose lower temperatures at which to spawn.

Introduction

Egg size in fish is the result of a spectrum of selection pressures operating on parents laying and caring for eggs, the eggs, and the resulting fry. The parent, for example, faces a tradeoff between egg size and egg number. The size of the egg influences the time it takes to hatch, an important consideration for an egg in risk of predation or falling water levels. Because a parent may be committed to parental care at least until the eggs hatch, the longer eggs take to develop, the greater the cost of parental care in terms of time and energy. Finally, the size of an egg may determine the size, health and capabilities of the fry and the duration of the post-hatch care. Understanding egg size is thus vital to unravelling the selection pressures influencing parental investment in fish.

The Cichlidae is one of the largest fish families, comprising some 105 genera (Nelson, 1994) and at least 1300 species, all of which provide some form of parental care, ranging from simple guarding to mouthbrooding. Within the Cichlidae, egg size is variable. The effective diameter (Coleman, 1991; in essence the three-dimensional average of the major and minor axes of nonspherical eggs) varies from 0.9mm to over 5.0mm. This represents a difference in egg volume of almost two orders of magnitude.

In this paper, I present a series of experiments and results focussing on Neotropical cichlids to elucidate the factors underlying the variation in egg size.

Experiment I: The extent of variation

How much variation is there in egg size? To examine the extent of variation in egg size amongst Neotropical cichlids, I have obtained either through breeding the fish myself, finding eggs in the wild, or by egg samples sent to me by other cichlid breeders, a large collection of cichlid eggs.

Methods

Eggs were preserved in either 70% isopropyl alcohol, or 4% formalin and then measured under a microscope using an ocular micrometer. Effective diameter was calculated as the cube-root of length x width x width.

Results

Egg size varyied from 0.9mm to 2.6mm, with most species falling in the range of 1.4 to 1.8 (Fig. 1).

Experiment II: The effect of temperature and egg size on hatching time

In theory, larger eggs should take longer to hatch because there is more material to convert from one state (the yolk) to another (the fry). In order to understand the effect of egg size on hatching time, it was first necessary to establish the relationship between temperature and hatching time because the effect of temperature is likely larger and must be controlled for in analyzing egg size.

Methods

Eggs from a single spawning were placed in "hatching cups" (the bottom portion of a plastic vial glued to a piece of glass) in aquaria maintained at different temperatures. The cups ensure that I can keep track of the eggs and yet allow good circulation of water. I put ten eggs into each cup, and then placed eight cups in each of six aquaria. Temperatures for the different aquaria ranged from 20 to 36°C.

Eggs were monitored around the clock for hatching. The "time-to-hatch" for a cup was taken as the time when 50% of the eggs in a cup had hatched. The scores for the eight cups were then averaged to give the hatching time for the particular temperature.

The experiment was repeated seventeen times using various species of Neotropical cichlids (convict Cichlasoma nigrofasciatum, midas C. citrinellum, rose-breast C. longimanus, rainbow Herotilapia multispinosa, bifasciatum C. bifasciatum, and tuba C. tuba. Species were chosen for their particular egg size.

Because hatching may not represent the same developmental stage in each of these different species, I chose to also evaluate the time to "swim-up", namely the point at which the fry can swim up off the bottom of the aquarium. This is a surprisingly distinct point in time. I recorded the data as that time at which 50% of the fry in an aquarium had reached swim-up.

Results

Temperature has a dramatic effect on hatching time and on swim-up time. For midas cichlids, at 34°C, the eggs hatch in a little over two days whereas at 22°C they take almost 5 days (Fig. 2).

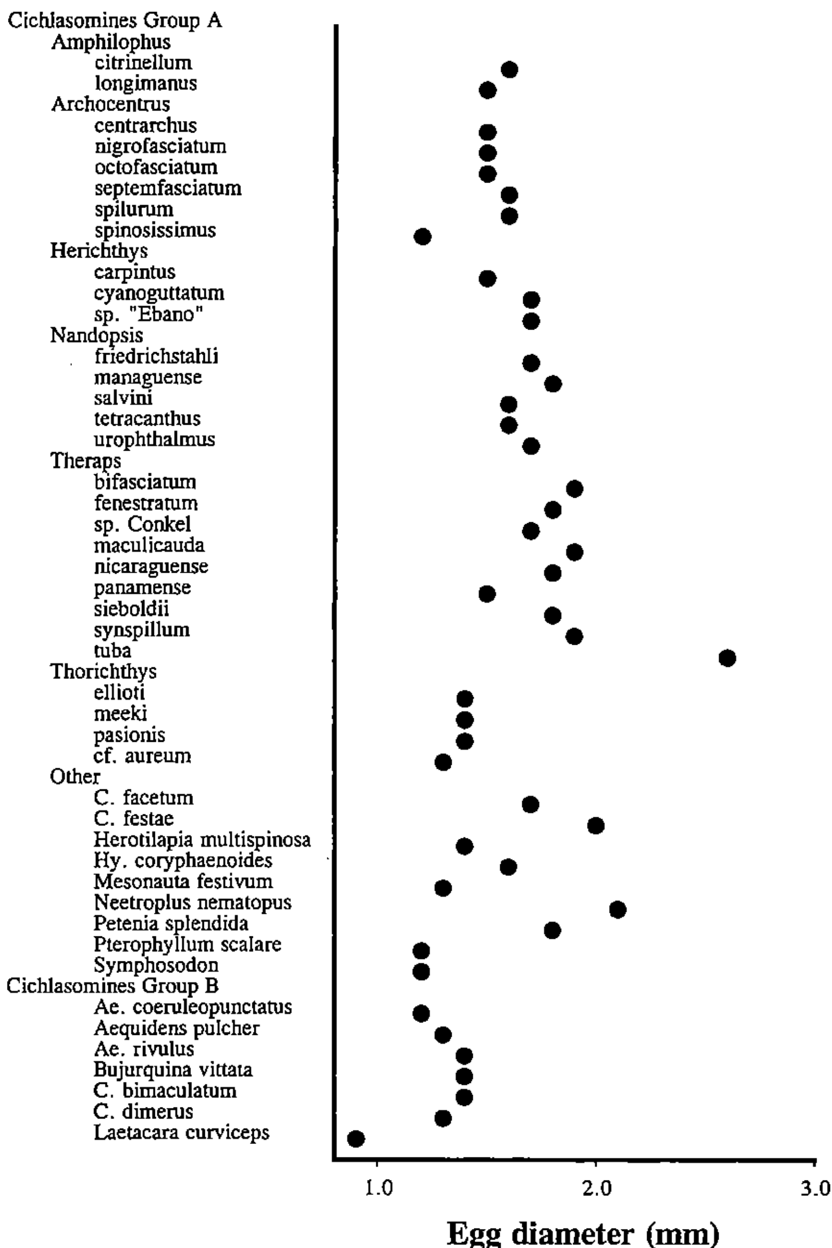

Figure 1. Egg size of forty-five species of Neotropical cichlids.

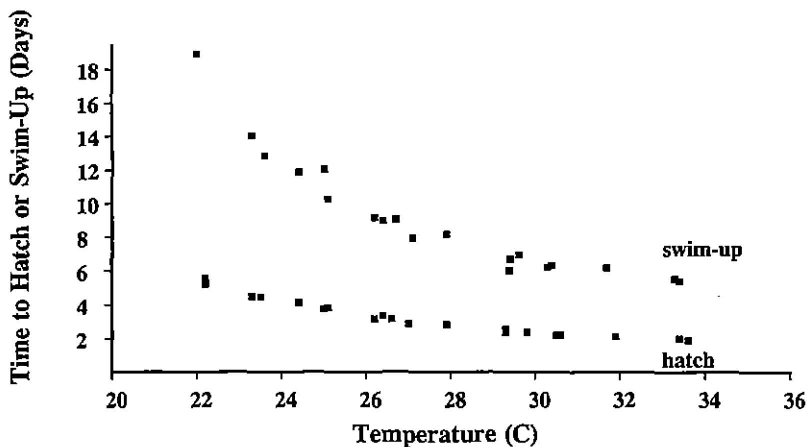

Figure 2. Development time versus temperature for midas

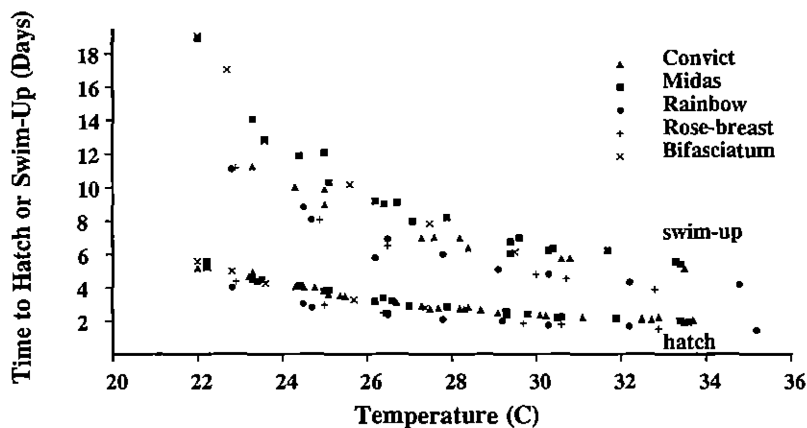

Figure 3. Development time for five cichlids

The effect of egg size is also present, though less clear (Fig. 3). Species with larger eggs, such as bifasciatum, midas and convict cichlids appear to take longer to develop than species with smaller eggs such as the rainbow cichlid. There is an exception, rose-breast cichlids, which have a larger egg, yet develop as quickly as those species with smaller eggs. This requires further investigation.

Experiment III - Temperature Choice

If temperature is so important to the development time in these cichlids, and consequently the duration of parental care, do parental cichlids actually choose the temperature at which they spawn? In the wild, they may have some opportunity to choose, either by not spawning during a certain season, or by choosing warmer or cooler parts of a river or lake.

Methods

To allow choice, I designed an aquarium with four chambers. Each was separated from the next chamber by a 3/4" plexiglass divider with a 2" x 4" notch cut in the bottom front corner. The thick plexiglass insulated each chamber from the next, while the notch allowed the fish to pass from one chamber to the next with a minimum of water flow. A bottomless flowerpot, lying on its side, was placed in each chamber as a potential spawning site.

Three aquarium heaters were inserted in the leftmost chamber, and the rightmost chamber was hooked up to a stock-tank continuously cooled by a chiller. This setup created a temperature gradient across the four chambers of as much as 10°C.

A pair of fish was placed in the apparatus and allowed to choose their spawning site, which they usually did within a few weeks. Three species were examined: convicts, rainbows and blue acaras (Aequidens pulcher).

Results

The results clearly indicate two points (Fig. 4). First, the different species appear to prefer slightly different temperatures to spawn at. Secondly, and perhaps most importantly, none of them prefer to spawn at the highest possible temperature as a general rule. Given the benefits demonstrated in the previous experiment, this seems puzzling.

Experiment IV - Temperature and Mortality

Parents may choose not to spawn at the warmest sites either because the eggs will not do well, or because the parents will not do well.

Methods

Using data gathered from the hatching experiment, I compared the mortality of eggs at different temperatures.

Results

While mortality is complete above 36°C and below 20°C, in between there is no clear relationship (Fig. 5). Thus, it is not egg mortality that drives the parental choice discovered in Experiment III.

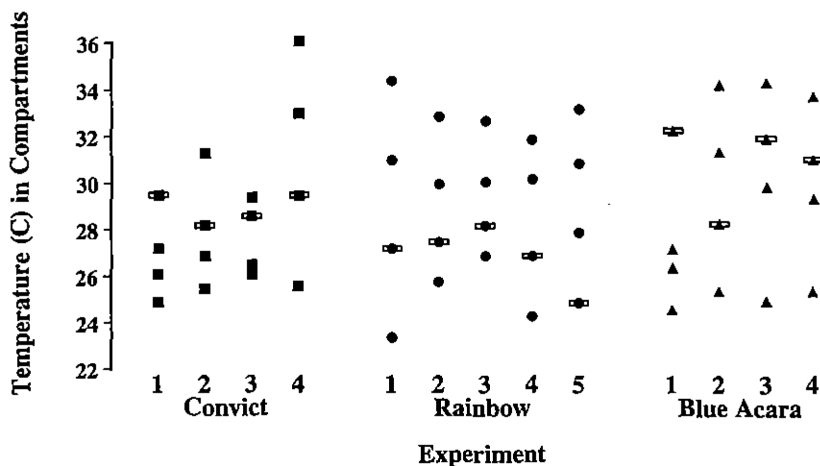

Figure 4. Spawning choice of three cichlids. The outline indicates which of the four compartments was chosen.

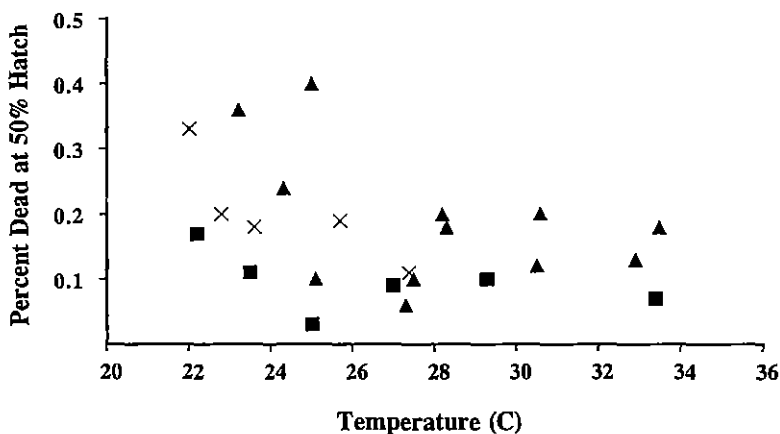

Figure 5. Mortality of eggs versus temperature.

Discussion

This series of experiments illustrates the extent of variation in egg size across a selection of Neotropical cichlids. The first experiment demonstrates substantial variation in egg size, with a few particularly noteworthy species with extreme egg size. For example, C. tuba has an unusually large egg, much larger than any other species. There are also species with tiny eggs. These species deserve particular attention in the future.

The tradeoff between egg size and egg number is well established elsewhere (Smith and Fretwell, 1974) so it is clear that parents pay a cost for having large eggs. There must be corresponding benefits. For example, Coleman and Galvani (this volume) have shown that larger eggs produce larger offspring. But, the question remains as to the exact advantages offered by larger offspring.

There are also additional costs of larger eggs. Experiment II illustrates that larger eggs take longer to hatch and longer still for the fry to reach the point of swimming. Since the parents must provide parental care at least to the point of free-swimming to ensure reasonable offspring survival, larger eggs mean an increase in the duration (and hence the cost) of parental care.

Experiment II also illustrated the profound effect of temperature on development time: higher temperatures dramatically shorten development time. Yet, Experiment III showed that parents in at least three species do not choose to spawn at the highest available temperature. Experiment IV showed that this is not because of increased offspring mortality at higher temperatures. Combined, these experiments suggest that it is something about the parents that prevents them from spawning at higher temperatures.

The most likely explanation for the puzzling choice of spawning temperatures is the cost of fanning. During the early stages of parental care, parental cichlids not only guard their offspring, but they must also fan the eggs to move oxygen-rich water over the eggs. Because warmer water contains less oxygen than cooler water, it seems likely that at warmer temperatures parental cichlids would have to fan faster than at cooler temperatures. Therefore I suggest that the species examined choose lower spawning temperatures because the cost of fanning would increase too much at higher temperatures. This is a testable prediction that I hope to examine in the near future.

Acknowledgements

I thank G.W. Barlow for use of his facilities, financial support and discussions. This research was supported in part by grant BNS 9109852 from the National Science Foundation to G.W. Barlow and from grants from the Guy Jordan Fund of the American Cichlid Association.

References

Coleman RM (1991) Measuring parental investment in nonspherical eggs. Copeia 1991:1092-1098

Nelson, JS (1994) Fishes of the World. Wiley, Toronto.

Smith CC and Fretwell SD (1974) The optimal balance between size and number of offspring. Am. Nat. 108:499-506.

FREE AMINO ACIDS AND PROTEIN CONTENT IN PELAGIC AND DEMERSAL EGGS OF TROPICAL MARINE FISHES

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Introduction

The content of free amino acids (FAA) in newly spawned marine fish eggs vary in proportion to egg size (Rønnestad & Fyhn, 1993). However, accumulating evidence suggests that the relative size of the FAA pool also is correlated to the spawning behaviour of the fish species, *i.e.* whether the eggs are demersal or pelagic. The pool of FAA in pelagic eggs seems to be markedly larger than that of demersal eggs. This observation may be related to the role of FAA in the process of final oocyte hydration; a function that appears to be more important in marine fishes with pelagic eggs (Thorsen & Fyhn, 1995). This final swelling of the oocyte seems to result from an osmotic water influx due to the hydrolysis of a specific yolk protein (Fyhn, 1993; Thorsen & Fyhn, 1995).
Most of our understanding of the roles served by FAA during fish embryogenesis is based on species from boreal waters (Rønnestad & Fyhn, 1993; Finn et al, 1995a,b). To increase the database in this area, a comparative study was therefore undertaken on newly spawned eggs of 23 species of marine tropical fishes belonging to 8 families from 5 different suborders of Perciformes. This report summarises the first results from the study.

Results and discussion

A comprehensive statistical evaluation of the data from the egg material is currently in progress. This analysis will also include other parameters (oil globule volume, yolk volume, dry weight, energy content, lipid classes and total fatty acids).

The data on FAA and protein contents (Table I, Figure 1) show that the pelagic eggs were characterised by a high total content of FAA, and with the pool profile spread out on several amino acids. The protein content was on average about 30% of the egg DW although the variation was substantial. The three species belonging to the suborders of Acanthuroidei and Percoidei had relative egg DWs which were markedly higher than that of the pelagic eggs from the suborder Labroidei. Differences in chorion solubility in the 1 M NaOH used in preparing the eggs for protein analysis, may partly be responsible for the variation in protein content among the pelagic eggs (Fyhn & Govoni, 1995). The FAA represented about a third of the total amino acid content (sum of protein-bound and free amino acids) in the pelagic eggs, thus supporting the notion that marine pelagic fish eggs in general contain a large pool of FAA (Rønnestad and Fyhn, 1993).

The demersal eggs showed a significantly higher protein content, about 45% of egg DW, but a FAA pool which ,in total, amounted to only about 8% of that in the pelagic eggs. The FAA

Table 1. Egg weight, free amino acid (FAA) and protein content of newly spawned eggs of 23 species of marine tropical fish all belonging to the order Perciformes. The data are presented as mean (± SD) of five batches from separate females.

Suborder	Family	Species	Egg	DW (µg/Ind)		total FAA (µmol/gDW)		total FAA (mg/gDW)		% of FAA Ess	% of FAA Non-ess	Protein (mg/gDW)		FAA % of tot AA*
Blennioidei	Blenniidae	*Ophioblennius atlanticus*	D	30,2	(2,3)	150,2	(15,8)	19,1	(1,8)	18,1	77,4	430,1	(62,3)	4,3
Gobioidei	Gobiidae	*Corypheopterus dicrus*	D	13,3	(1,1)	94,0	(22,1)	12,0	(2,8)	20,9	75,9	492,1	(61,6)	2,4
Gobioidei	Gobiidae	*Coryphopterus personatus*	D	9,6	(0,4)	121,8	(15,8)	15,4	(2,0)	16,3	81,6	518,5	(39,2)	2,9
Percoidei	Pomacentridae	*Abudefduf saxatilis*	D	47,5	(3,5)	66,0	(16,3)	8,5	(2,5)	14,3	82,2	509,2	(103,2)	1,7
Percoidei	Pomacentridae	*Abudefduf troschelii*	D	36,6	(2,4)	42,9	(10,0)	4,2	(2,4)	13,2	76,3	495,0	(26,7)	1,1
Percoidei	Pomacentridae	*Chromis atrilobata*	D	14,9	(1,4)	47,9	(19,5)	5,3	(2,2)	8,7	86,8	524,3	(27,5)	1,0
Percoidei	Pomacentridae	*Chromis multilineata*	D	18,6	(1,4)	71,3	(10,5)	8,2	(1,1)	10,8	87,7	498,1	(36,4)	1,6
Percoidei	Pomacentridae	*Microspathodon chrysurus*	D	28,8	(1,2)	93,1	(12,3)	12,4	(1,4)	10,3	89,8	429,0	(9,3)	2,8
Percoidei	Pomacentridae	*Stegastes planifrons*	D	27,1	(6,0)	139,2	(28,3)	17,7	(3,7)	20,8	79,2	391,8	(17,2)	4,3
Percoidei	Pomacentridae	*Stegastes variabilis*	D	29,5	(3,2)	113,2	(24,9)	14,6	(3,3)	16,3	83,7	380,8	(25,8)	3,7
Acanturoidei	Acanthuridae	*Acanthurus coeruleus*	P	11,3	(0,1)	900,3	(165,0)	112,2	(21,1)	49,4	50,1	377,5	(5,6)	22,9
Percoidei	Chaetodontidae	*Chaetodon capistratus*	P	14,3	(2,3)	712,8	(322,6)	59,0	(55,3)	50,0	49,6	426,5	(49,6)	18,0
Percoidei	Haemulidae	*Haemulon flavolineatum*	P	24,3	(0,6)	263,9	(200,5)	33,1	(24,8)	50,7	47,7	542,9	(29,4)	5,6
Labroidei	Labridae	*Bodianus diplotaenia*	P	13,3	(1,9)	1 458,9	(227,7)	186,3	(29,6)	56,4	43,3	215,1	(38,7)	47,2
Labroidei	Labridae	*Bodianus rufus*	P	19,9	(0,5)	1 518,2	(7,8)	193,4	(8,3)	55,6	44,1	301,0	(27,0)	39,2
Labroidei	Labridae	*Clepticus parrae*	P	13,4	(1,6)	1 321,1	(188,0)	169,4	(23,3)	55,3	44,2	280,9	(35,7)	37,7
Labroidei	Labridae	*Halichoeres bivittatus*	P	8,3	(1,4)	1 150,5	(132,3)	148,5	(17,7)	57,2	42,8	210,6	(15,1)	41,4
Labroidei	Labridae	*Halichoeres garnoti*	P	11,6	(0,9)	1 233,1	(117,0)	158,5	(15,2)	57,9	41,8	298,3	(24,9)	35,6
Labroidei	Labridae	*Halichoeres poeyi*	P	8,4	(2,2)	1 154,2	(88,8)	150,5	(10,4)	57,4	41,8	235,1	(15,2)	39,5
Labroidei	Labridae	*Thalassoma bifasciatum*	P	4,6	(0,4)	1 396,4	(56,5)	178,9	(9,1)	55,2	44,0	194,6	(10,0)	47,9
Labroidei	Scaridae	*Scarus iserti*	P	12,5	(1,5)	1 178,3	(53,6)	125,8	(62,0)	52,3	47,3	235,7	(17,2)	39,1
Labroidei	Scaridae	*Sparisoma aurofrenatum*	P	17,8	(8,2)	1 499,9	(140,4)	161,5	(80,7)	52,0	48,0	215,4	(26,4)	47,4
Labroidei	Scaridae	*Sparisoma rubripinne*	P	11,3	(1,6)	1 264,1	(76,3)	165,6	(9,7)	52,6	47,4	224,6	(10,7)	42,4

D: Demersal eggs
P: Pelagic eggs
**: FAA as % of total amino acids (AA: free+ protein amino acids)

comprised only 2-3% of the total amino acid content in the demersal eggs, and the pool profile was strongly dominated by a single amino acid. These findings are in line with earlier observations on demersal fish eggs from boreal waters (Thorsen, 1995).

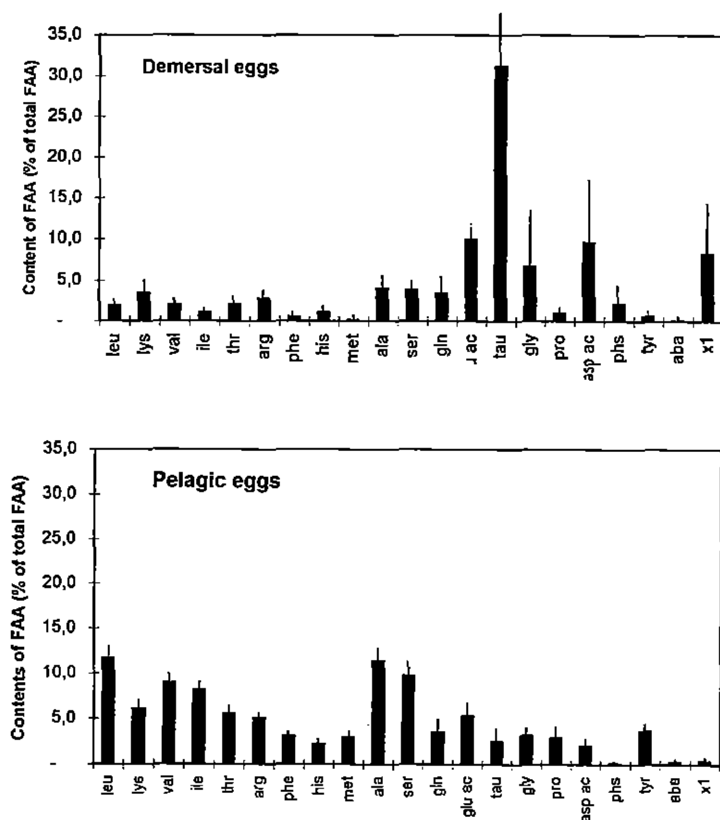

Figure 1. Average free amino acid content of newly spawned demersal and pelagic eggs of 23 species of marine tropical fish all belonging to the order Perciformes.

The FAA profiles (Fig. 1) showed the pelagic eggs to have similar pool composition and to be in agreement with the previously described profile of pelagic eggs from boreal fishes (Rønnestad and Fyhn, 1993). Essential and non-essential amino acids made up about equal proportions of the pool. Leucine, valine, and isoleucine dominated among the essential amino acids whereas alanine and serine dominated among the non-essentials. The similarity of the FAA profile among all teleosts tested so far suggests that this reflects an phylogenetically ancient and central process in their adaptation to life in seawater. The FAA profile of the demersal eggs differed strongly from that of the pelagic eggs. Moreover, the demersal eggs had a more variable FAA pool, and essential amino acids accounted only for about 15% of the FAA. Taurine which is not incorporated into proteins, was the dominating amino acid in the FAA pool of the demersal eggs suggesting influx and not proteolysis to be the mechanism of accumulation in these eggs before spawning.

Conclusions

The notion that pelagic marine fish eggs contain a large pool of FAA is further strengthened, and pelagic eggs of tropical fishes are not different from those of boreal fishes in this respect. The similarity of the FAA pool in these eggs supports the idea that the pool originates from hydrolysis of a single yolk protein, and that this is a primitive trait among marine fishes, central in their adaptation to life in the sea. The difference between pelagic and demersal eggs with respect to FAA and protein content is so clear cut that it can be used as a diagnostic tool to discriminate between randomly collected eggs of the two types.

Acknowledgments

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Literature cited

Finn, RN, Fyhn, HJ Evjen MS 1995 Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod Gadus morhua). I. Respiration and nitrogen metabolism. Marine Biology 124: 355-369

Finn, RN, Rønnestad, I, Fyhn HJ 1995 Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut *(Hippoglossus hippoglossus).* Comparative Biochemistry and Physiology. 111A: 647-671

Fyhn, HJ 1993. Multiple functions of free amino acids during embryogenesis in marine fishes In:, In: Walther, B.T. and H.J. Fyhn (eds.) "Physiology and Biochemistry of marine fish larval development". University of Bergen, Bergen, Norway. Pp. 285-289

Fyhn, HJ, Govoni, JJ 1995. Endogenous nutrient mobilization during egg and larval development in two marine fishes- Atlantic menhaden and spot. ICES mar. Sci. Symp. 210: 64-69

Rønnestad, I, Fyhn HJ 1993 Metabolic aspects of free amino acids in developing marine fish eggs and larvae. Reviews in Fisheries Science 1 (3): 239-259

Thorsen, A 1995. Oogenesis in marine bony fishes: Physiological mechanisms of oocyte hydration and egg buoyancy Dr. thesis. University of Bergen, Bergen, Norway

Thorsen, A, Fyhn, HJ 1995. Final oocyte maturation in vivo and in vitro in marine fishes with pelagic eggs; yolk protein hydrolysis and free amino acid content. Journal of fish Biology (In Press)

SYNTHESIS OF FREE AND CONJUGATED 17α,20β-DIHYDROXY-4-PREGNEN-3-ONE

BY EMBRYOS OF ARCTIC CHARR *(Salvelinus alpinus)*

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Introduction:

The yolk of the eggs of several fish species has been shown to contain significant levels of hormones, including the progestogen, 17α,20β-dihydroxy-4-pregnen-3-one (17,20P) that has been identified in the eggs of coho salmon, *Oncorhynchus kisutch* (Feist et al., 1990), steelhead trout, *Oncorhynchus mykiss* (Yeoh 1993) and Arctic charr, *Salvelinus alpinus* (Khan et al., 1995). Although the role of this steroid hormone in final oocyte maturation in salmonids is well established (see Nagahama, 1994 for review), its possible role in the early development of the fish embryo is not known. Because of their actions on gene expression, steroid hormones that are present in the yolk could have a potentially significant role in early embryonic development; some authors have argued that these hormones may play a central regulatory role prior to the emergence of the embryo's own endocrine system (Schreck et al., 1991; Lam, 1994).

Changes in free steroid hormone profiles during early ontogeny have been reported in several species of fish (rainbow and steelhead trout: Antila, 1984, Yeoh 1993; tilapia, *Oreochromis nilotica*: Rothbard et al., 1987; coho salmon: Feist et al., 1990). Such changes are indicative of active steroid hormone metabolism and/or excretion. Indeed, Yeoh (1993) provided direct evidence of metabolism of [^{3}H]pregnenolone to free androstenedione (A_4), 17β-estradiol (E_2) and testosterone (T) by embryonic and larval steelhead trout; in addition, the authors found glucuronide forms of T and E_2. Venkatesh et al. (1991) also reported that embryos of guppy (*Poecilia reticulata*) were able to convert the steroid precursors into various metabolites, including glucuronide forms. The conjugation of steroid hormones is usually considered as the first step in the process of elimination (excretion) of these hormones (Iwata et al., 1994). Excreted steroid hormone sulphates, in particular 17α,20β-dihydroxy-4-pregnen-3-one-20-sulphate (17,20P sulphate), and glucuronides are also thought to be used as pheromones (Colombo et al., 1980; Van den Hurk and Lambert, 1983; Resink et al., 1989; Scott and Vermeirssen 1994; Sorensen et al., 1995; Scott et al., 1995).

Previously, the free form of 17,20P was thought to be produced only during late oocyte maturation it has been termed maturation inducing hormone (MIH). More recently, this hormone

has also been shown to be synthesized by the testicular germ cells of immature rainbow trout (Vizziano et al., 1995, 1996) and by spermatid of the roach, *Rutilus rutilus* (Ebrahimi et al. 1995). There are no reports of synthesis of 17,20P by embryonic tissues of fish; here we present the first evidence of the synthesis of 17,20P and its conjugated metabolites by teleost embryos. We incubated Arctic charr embryos *in vitro* with a radiolabelled steroid hormone precursor [^{3}H]17α-hydroxyprogesterone (17αOHP) and employed HPLC methods to identify the radiolabelled metabolic products. The objective was to determine if Arctic charr embryos are able to metabolize 17αOHP at various stages of their ontogenetic development.

Materials and methods:

In vitro incubation

"Eyed" and newly hatched yolksac embryos of Arctic charr [28 and 62-74 days post fertilization (dpf), respectively] were used in the study. The embryos were killed by decapitation and the chorion and yolksac was removed from each embryo where applicable, and the whole embryo was incubated. All dissections were carried out on ice-chilled trays, after which the incubations were carried out at 10°C. Three animals (approximately 50-70 mg in weight) were placed in individual tissue culture wells (24-well plate, 3.5 ml well capacity, M/S Flow Laboratories Inc., VI, USA). Each well contained 2 ml of modified Medium 199 (M-3274 M/S Sigma Chemical Company) with Hank's salts, glutamine, sodium bicarbonate, bovine albumin, antibiotics and glucose, without phenol red, pH 7.2 (Venkatesh et al., 1992a). The radiolabelled substrate [^{3}H]17αOHP was added to each well (8 nmol; 0.8μCi) at the beginning of the incubation period. Incubation periods of 20 min, 60 min, 12 h and 24 h were used; the contents of each well (tissue and medium) were stored frozen at -20°C until analysed for steroid hormone content.

Extraction of steroid hormones and HPLC

The extraction procedure for HPLC was based on the methods described by Payne et al. (1989), Venkatesh et al. (1989), Kime et al. (1991) and Scott and Canario (1992) with some modifications. The incubate and embryonic tissues were applied directly to a Sep-Pak C_{18} column which had been primed by washing with 5 ml of methanol and 5 ml of distilled water. The incubate was passed through the Sep-Pak column by syringe and the column was again washed with 5 ml of water and 5 ml of hexane. Subsequently, the free steroid hormones were washed from the column using 5 ml of diethyl ether and the conjugated steroids were eluted with 5 ml of 100 % methanol. The washed embryos carcases retained on the cartridge were removed after the first wash. The ether and methanol extracts were dried separately under nitrogen at 45°C.

The free fraction containing radiolabelled metabolites was dissolved in 200 μl of acetonitrile containing a mixture of 21 unlabelled steroid hormones (as internal standards); 20 μl of this mixture was injected onto the HPLC column. The methanol fraction containing conjugated steroid hormones was processed for acid solvolysis and enzyme hydrolysis.

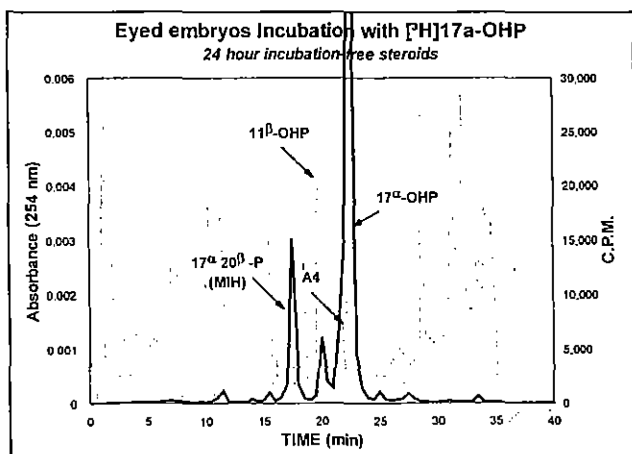
The HPLC apparatus consisted of a Waters Associates (Milford, MA, USA) Liquid Chromatography System comprising two 510 pumps, a WISP 710B autoinjector and a solvent programmer (Waters Baseline 810 controller Software), coupled with a dual channel model 441 UV detector. The NOVA.Pak C18 (8 mm X 100 mm, 4 μm) column was also purchased from Waters Associates. For optimum separation of steroid hormones, Waters Baseline 810 controller Software was used to create a binary solvent gradient of acetonitrile-water. The multistep gradient

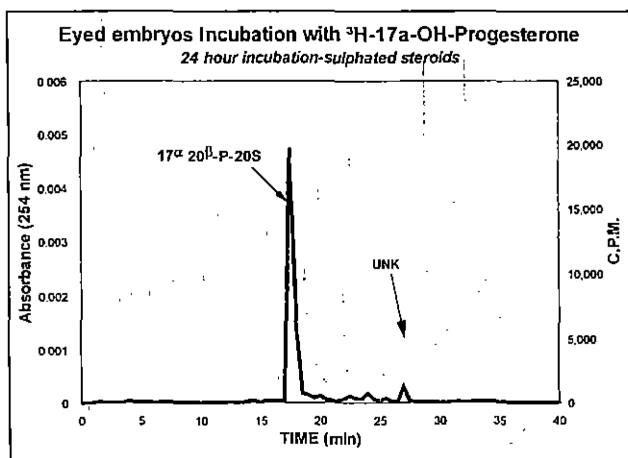
mobile phase consisted of 29.5, 35, 39, and 75% acetonitrile in water at 0, 10, 12.5 and 32 min, respectively. In order to obtain better yield of metabolites from the HPLC column, the incubation replicates were pooled together after extraction, in acetonitrile containing 21 steroid standards and injected into column as described above. Retention times for each steroid hormone was determined from the elution time of the internal standards and the absorbance was monitored at 254 and 280 nm. The counts (CPM) in each fraction and absorbance of 21 internal standards was plotted against retention time.

Results:

All three developmental stages examined in this study (28, 62 and 74 dpf) exhibited essentially similar steroid metabolite profiles following incubation with [^{3}H]17αOHP, and in all three stages the conversion of radiolabelled 17αOHP into radiolabelled metabolites was progressive, beginning within 20 min of the addition of [^{3}H]17αOHP. For expediency, representative chromatographs of 28 dpf embryos incubated for 24 h data are shown (Figs. 1 and 2).

The major radiolabelled peaks of free steroid hormones produced following incubation of all three embryo groups with [^{3}H]17αOHP co-eluted with 17,20P and androstenedione (A_4); these peaks represented between 13.2 and 16.9% and 1.5 and 5.0%, respectively of the "initial" [^{3}H]17αOHP radioactivity counts in the 24 h incubations (Table 1). The identification of the A_4 peak is made tentatively because of its close elution with the major radiolabelled precursor, 17αOHP (Fig.1). A second smaller peak co-eluted close to the same time as 11ßOHP (Fig. 1).





Figures 1 (upper) and 2 (lower). HPLC profiles of radiolabelled free (Fig. 1) and sulphated (Fig. 2) steroid hormones produced by Arctic charr embryos after incubation for 24 h with [^{3}H]17α-hydroxyprogesterone ([^{3}H]17-OHP). 17,20-P = 17α,20ß-dihydroxy-4-pregnen-3-one (MIH = "maturation" inducing hormone); 11ß-OHP = 11ß-hydroxyprogesterone; A_4 = androstenedione; 17α,20ß-P-20S = 17α,20ß-dihydroxy-4-pregnen-3-one 20 sulphate; UNK = unknown metabolite.

Table 1.
Metabolism of [^{3}H]17α-hydroxyprogesterone ([^{3}H]17αOHP) into free and conjugated steroids by eyed (28 dpf), and hatched embryos (62 & 74 dpf) of Arctic charr after 20 min and 24 h incubation at 10°C in modified Medium 199 containing 8.0 nmol per well of [^{3}H]17αOHP as substrate. The radioactivity (CPM) measured under the respective peak area and expressed as % of the counts in the substrate peak at 20 min of incubation. 17,20P = 17α,20β-dihydroxy-4-pregnen-3-one (MIH = "maturation" inducing hormone); A_4 = androstenedione; 17,20P-SO4 = 17,20P-sulphate; 17,20P-gluc. = 17,20P-glucuronide.

		Age of embryos		
Metabolites	**Time**	**28 dpf**	**62 dpf**	**74 dpf**
17αOHP (substrate)	20 min	100	100	100
	24 h	60.5	35.6	35.9
17,20P (MIH)	20 min	2.1	3.3	2.0
	24 h	11.4	16.9	13.2
A_4	20 min	6.3	2.7	16.6
	24 h	5.0	1.5	2.1
17,20P-SO_4	20 min	0.5	0.9	0.5
	24 h	18.0	30.2	17.6
17,20P-Gluc.	20 min	<0.1	0.1	<0.1
	24 h	0.8	1.5	0.6

There were time-dependent decreases in the radiolabel associated with the free steroid hormone fraction for all three ages of embryos, and concomitant increases in radiolabel present in the sulphate- and glucuronide-conjugated steroid fractions (Table 2). However, the appearance of glucuronide forms was considerably less than that of the sulphated forms (Table 2).

The major radiolabelled conjugated form of steroid hormone co-eluted with the unlabelled 17,20P standard for both sulphates (Fig. 2; Table 2) and glucuronides (chromatograph not shown; Table2). Small unknown radiolabelled peaks were also found at 27.0 min and 24.0 min for the sulphated and glucuronide forms, respectively (Fig. 2).

Table 2.
Metabolism of [^{3}H]17α-hydroxyprogesterone ([^{3}H]17αOHP) into free and conjugated steroids by eyed (28 dpf), and hatched embryos (62 & 74 dpf) of Arctic charr after 20 min, 1 h, 12 h, and 24 h incubation at 10°C in modified Medium 199 containing 8.0 nmol per well of [^{3}H]17αOHP as substrate. The radioactivity (CPM) measured in 100 µl of ether extract in respective free, sulphated and glucuronide fractions.

Age (dpf)	Time	Free	Sulphate	Glucuronide
28[a]	20 min	98.37 ± 0.20	1.30 ± 0.18	0.32 ± 0.03
	1 h	97.25 ± 0.61	2.35 ± 0.54	0.38 ± 0.06
	24 h	81.26 ± 0.98*	17.56 ± 1.05*	1.15 ± 0.08*
62	20 min	96.76 ± 0.18	2.69 ± 0.09	0.53 ± 0.14
	1 h	96.94 ± 0.23	2.75 ± 0.30	0.29 ± 0.11
	12 h	71.10 ± 3.36*	26.51 ± 3.97*	2.25 ± 0.29*
	24 h	63.49 ± 4.53*	32.58 ± 3.99*	3.90 ± 1.00
74	20 min	94.91 ± 1.15	4.40 ± 0.97	0.66 ± 0.21
	1 h	93.20 ± 2.19	4.03 ± 0.42	2.74 ± 2.14
	12 h	83.94 ± 2.10*	13.24 ± 1.73*	2.80 ± 0.37*
	24 h	73.98 ± 3.76*	22.60 ± 3.28*	3.39 ± 0.48*

[a]12 h incubation data not taken; mean ± SEM; n = 3; * significantly different from 20 min of respective incubations ($p < 0.05$).

Discussion:

These incubation studies show that early "eyed" and hatched embryos of Arctic charr are capable of synthesizing 17,20P and its sulphated and glucuronidated conjugates from 17OHP. The formation of 17,20P as a major metabolite of 17OHP metabolism has been widely reported in various *in vitro* studies, in which ovarian follicles of several fish species were incubated with substrate [goldfish: Kime et al. (1992); guppy: Venkatesh et al., (1992b); European catfish (*Silurus glanis*): Kime et al. (1993); Indian catfish (*Clarias batrachus*): Haider and Balamurugan (1995)]. However, to our knowledge this report is first to provide evidence of the synthesis of 17,20P by embryos of any fish, and indicates that early embryonic tissues of Arctic charr already express 20β-HSD. Arctic charr embryos seem to have a preference for the formation of 17,20P over A_4 (the latter is formed only in trace quantities as a smaller peak forming the shoulder of the large substrate 17αOHP peak) (Fig. 1). It is possible that the enzyme responsible for conversion of 17αOHP to A_4 on the main steroidogenic pathway [17,20 desmolase (lyase)] is not sufficiently expressed in embryos to convert all the substrate to A_4, thus causing a shift towards the formation of 17,20P. Conversely, it could also be argued that the insufficient expression of 17,20 desmolase

(thereby ignoring the main inactivation pathway) and the preferred production of 17,20P rather than the relatively "safer" A_4 is an indication that 17,20P has some as yet unknown physiological value for the embryo possibly affecting gonadal development and differentiation, feedback regulation of the pituitary and/or playing an autocrine/paracrine function.

Our study suggests that 20β-HSD is expressed (maybe transiently) early in embryogenesis and it is likely that the production site is extragonadal. Unlike the situation in the adults, where MIH synthesis by the ovary is regulated by pituitary GtH II (Nagahama, 1994), the synthesis of 17,20P in the embryo is probably independent of a central feedback regulation.

The formation of sulphated and glucuronated forms of 17,20P in Arctic charr embryos is indicative of a secondary route for the metabolism of this steroid, whereby newly-formed 17,20P acts as substrate for the sulphuryl- and glucuronyl transferases; the embryos appear to have a preference for sulphated conjugation as compared to glucuronidation for 17,20P. It is possible that the sulfotransferase is more efficient than the UDP glucuronyl transferase, thereby resulting in higher yield of sulphated steroids than glucuronated steroid hormones. The expression of these enzymes in fish embryos may provide the means by which the embryo protects itself from the effects of steroids, by facilitating their excretion. If the embryo is likely to be exposed to unregulated sources of steroid hormones, there is a clear biological value in maintaining an ability to rapidly metabolize and/or excrete these biologically active materials.

References:

Antila, E. (1984). Steroid conversion by oocytes and early embryos of *Salmo gairdneri*. Ann. Zool. Fenn. 21, 465-471.

Colombo, L., Marconato, A., Colombo Belvedere, P., and Frisco, C. (1980). Endocrinology of teleost reproduction: a testicular steroid pheromone in the black goby. Boll. Zool. 47,355-364.

Ebrahimi, M., Singh, P.B., and Kime D.E. (1995). Biosynthesis of 17,20α-dihydroxy-4-pregnen-3-one, 17,20β-dihydroxy-4-pregnen-3-one, and 11-ketotestosterone by testicular fragments and sperm of the Roach, *Rutilus rutilus*. Gen. Comp. Endocrinol. 100, 375-384.

Feist, G., Schreck, C.B., Fitzpatrick, M.S., and Redding, J.M. (1990). Sex steroid profiles of coho salmon (*Oncorhynchus kisutch*) during early development and sexual differentiation. Gen. Comp. Endocrinol. 80, 299-313.

Haider, S., and Balamurugan, K. (1995). Presence of maturation-promoting factor in 17α, 20β-dihydroxy-4-pregnen-3-one-induced oocyte of catfish, *Clarias batrachus*. Fish Physiol. Biochem. 14, 501-508.

Iwata, T.I., Hirose, T., Nakamura, M., and Yamaguchi, M. (1994). Determination of urinary glucuronide conjugates by high-performance liquid chromatography with pre-column fluorescence derivatization. J. Chromatogr. B 654, 171-176.

Johnstone, R., Macintosh, D.J., and Wright, R.S. (1983). Elimination of orally administered 17α-methyltestosterone by *Oreochromis mossambicus* (tilapia) and *Salmo gairdneri* (rainbow trout) juveniles. Aquaculture 35, 249-257.

Khan, M.N., Renaud, R., and Leatherland, J.F. (1995). Correraltion between plasma and egg steroid hormone content of Arctic charr. In: Proc. Vth Int. Symp. Rep. Physiol. Fish, Austin Texas, F.W. Goetz and P. Thomas (Eds.), 366.

Kime, D.E., Venkatesh, B., and Tan, C.H. (1991). 5α-Pregnane-3β,7α,17,20α- and -20β-tetrols as metabolites of progesterone and 17-hydroxyprogesterone in carp (*Cyprinus carpio*) ovarian incubations. Gen. Comp. Endocrinol. 84, 401-404.

Kime, D.E. Bhattacharya, S., Koldras, M., and Bieniarz, K. (1993). Steroidogenesis by ovaries and testes of the European catfish, the wels (*Silurus glanis*), *in vitro*. Fish Physiol. Biochem. 10, 389-398.

Kime, D.E., Scott, A.P., and Canario, A.V.M. (1992). *In vitro* biosynthesis of steroids, including 11-deoxycortisol and 5α-pregnane-3β,7α,17,20β-triol, by ovaries of the goldfish *Carassius auratus* during the stage of oocyte final maturation. Gen. Comp. Endocrinol. 87, 375-384.

Lam, T.J. (1994). Hormones and egg/larval quality in fish. J. World Aqualt. Soc. 25, 2-12.

Nagahama, Y. (1994). Endocrine regulation of gametogenesis in fish. Int. J. Dev. Biol. 38, 217-229.

Payne, D.W., Holtzclaw, W.D., and Adashi, E.Y., (1989). A convenient unified scheme for the differential extraction of conjugated and unconjugated serum C_{19} steroids on Sep-Pak C_{18}-cartridges. J. Steroid Biochem., 33, 289-295.

Resink, J.W., Voorthuis, P.K., Van den Hurk, R., Peters, R.C., and Van Oordt, P.D.W.J. (1989). Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish, *Clarias gariepinus*. Aquaculture 83, 153-166.

Rothbard, S., Zohar, Y., Zmora, N. , Levavi-Sivan, B., Moav, B., and Yaron, Z. (1990). Clearance of 17α-ethynyltestosterone from muscle of sex-reveresd tilapia hybrids treated for growth enhancement with two doses of the androgen. Aquaculture 89, 365-376.

Schreck, B.S., Fitzpatrick, M.S., Feist, G.W. and Yeoh, C.G. 1991. Steroids: Developmental continum between mother and offspring. In: Proc. IVth Int. Symp. Rep. Physiol. Fish, Sheffield, England, A.P.Scott, J.P. Sumpter, D.E.Kime and M.S. Rolfe (Eds.), 256-258.

Scott, A.P., and Vermeirssen, E.L.M. (1994). Production of conjugated steroids by teleost gonads and their role as pheromones. In: Perspectives in Comparative Endocrinology. National Research Council of Canada, Ottawa, K.G. Davey, R.E. Peter and S.S. Tobe (Eds.), 645-654.

Scott, A.P., and Canario, A.V.M. (1992). 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate: A major new metabolite of teleost oocyte maturation-inducing steroid. Gen. Comp. Endocrinol. 85, 91-100.

Scott, A.P., Nagahama, Y., Van Der Kraak, G., and Nagler, J.J. (1995). Sulfation and uptake

of the maturation-inducing steroid, 17α,20β-dihydroxy-4-pregnen-3-one by rainbow trout ovarian follicles. Fish Physiol. Biochem. 14, 301-311.

Sorensen, P.W., Scott, A.P., Stacey, N.E., and Bowdin, L. (1995). Sulfated 17,20β-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. Gen. Comp. Endocrinol. 100, 128-142.

Van den Hurk, R., and Lambert, J.G.D. (1983). Ovarian steroid glucuronides function as sex pheromones for male zebrafish (*Brachydanio rerio*). Can. J. Zool. 61, 2381-2387.

Venkatesh, B., Tan, C.H., Kime, D.E., and Lam, T.J. (1992a). Steroid metabolism in teleost gonads: Purification and identification of metabolites by high-performance liquid chromatography. Steroids 57, 276-281.

Venkatesh, B., Tan, C.H., Kime, D.E., and Loy, G.L. (1991). Steroid metabolism and synthesis of highly polar 7-hydroxylated steroids by ovarian follicles and extrafollicular tissue of guppy (*Poecilia reticulata*) during oocyte growth and gestation. In: Proc. IVth Int. Symp. Rep. Physiol. Fish, Norwich, UK, A.P. Scott, J.P. Sumpter, D.E. Kime, and M.S. Rolfe (Eds.), 105.

Venkatesh, B., Tan, C.H., Kime, D.E., Loy, G.L., and Lam T.J. (1992b). Steroid metabolism by ovarian follicles and extrafollicular tissue of guppy (*Poecilia reticulata*) during oocyte growth and gestation. Gen. Comp. Endocrinol. 86, 378-394.

Venkatesh,B., Tan, C.H., and Lam, T.J. (1989). Blood steroid levels in the goldfish: measurement of six ovarian steroids in small volumes of serum by reverse-phase high-performance liquid chromatography and radioimmunoassay. Gen. Comp. Endocrinol. 76, 398-407.

Vizziano, D., Le Gac, F., and Fostier, A. (1995). Synthesis and regulation of 17α-hydroxy-20β-dihydroprogesterone in immature males of *Oncorhynchus mykiss*. Fish Physiol. Biochem. 14, 289-299.

Vizziano, D., Fostier, A., Le Gac, F., and Loir, M. (1996). 20β-hydroxysteroid dehydrogenase activity in nonflagellated germ cells of rainbow trout testis. Biol. Reprod. 54, 1-7.

Yeoh, C.G. (1993). The effects of hormones on development of embryonic and post embryonic salmonids, and hormone metabolism during these stages. (M.Sc Thesis Oregon State University, USA).

LIPID AND PROTEIN CHANGES DURING EMBRYO DEVELOPMENT IN THE VIVIPAROUS GENUS SEBASTES: APPLICATION TO THE ASSESSMENT OF REPRODUCTIVE SUCCESS

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Abstract

Concentrations of total lipid, protein, and lipid classes were determined in oocytes and embryos from two species of viviparous rockfishes; yellowtail rockfish, *Sebastes flavidus*, and shortbelly rockfish, *Sebastes jordani*. Total lipid, protein, triacylglycerols, sterol/wax esters, nonesterified fatty acids, cholesterol, and polar lipids declined during embryo development. Regression analysis of changes in lipid and protein variables depending on the stage of embryo maturation found highly significant linear relationships. By solving regression equations for the final stage of embryo development, estimates of the concentrations of lipids and protein at birth were obtained. These values provide an estimate of the nutritional and structural biochemical composition of larvae at their earliest life history stage in the environment, and thus, an assessment of larval condition and reproductive quality. Data revealed significant differences in the dynamics of lipid and protein depletion during embryogenesis between the two rockfish species and among three populations of shortbelly rockfish located at Ascension, Pioneer, and Bodega submarine canyons. Estimated concentrations of lipids and protein at birth were significantly greater in shortbelly rockfish larvae from Ascension Canyon than in those from Pioneer and Bodega Canyons in 1994, suggesting a greater potential survival during the time period following birth when planktonic food resources are often limited. The technique presented here to assess reproductive quality or the condition of progeny can be applied in field studies of viviparous teleosts and does not require detailed laboratory investigations of embryo development.

Introduction

The assessment of reproductive success includes the determination of both the quantity and quality of progeny. Year-class, or spawn, strength may be influenced by the health of newborns as well as the number produced. The number of offspring produced, or fecundity, is often used as a measure of reproductive success in fish research, and has been related to biological and environmental factors (Blaxter, 1969). Less attention has been given to the assessment of the quality of reproductive

output. This is due primarily to the difficulty of determining which variables or processes are valid measures of egg, embryo, or larval health. Various measures have been proposed, such as egg size (Blaxter and Hempel, 1963), histological criteria (Theilacker, 1978), and biochemical analyses, including nucleic acids (Buckley, 1984), enzyme activity (Clarke et al., 1992), and biochemical composition (reviewed in Ferron and Leggett, 1994). All have merits justifying their use; however, other factors, such as clear relationships to growth or survival potential, ease and/or cost of analysis, or the ability to assess adequate numbers of replicates for statistical validity, often diminish their utility in routine assessment of reproductive success. Consideration of when during egg or embryonic development valid estimates can be made is also critical to accurate assessment of success. Ideally, evaluation at hatching, or birth, would provide the most accurate determination of the health and survival potential, but this stage is of very short duration and obtaining an adequate number of individuals to characterize a population or species is very difficult.

The determination of reproductive success in marine fishes, especially those that reproduce far from shore, is further complicated by the difficulty and cost of obtaining sufficient numbers of gravid females, or their eggs or embryos. In viviparous species, the complete development from previtellogenic oocyte to hatched larva occurs within the female. This simplifies collection of samples since only one life stage, adult, and not planktonic specimens needs to be evaluated.

We present here a technique that allows estimation of the nutritional status of hatched larvae at parturition, or release into the environment. Nutritional status has a clear relationship to growth and survival potential in that the amount of metabolically available energy establishes the duration of survival in the environment until adequate food becomes available. The method was applied to two species in the viviparous genus *Sebastes*, a taxon well-represented in the northeastern Pacific and commercially and recreationally important from Alaska to Baja California. The assessment of nutritional status involved the determination of protein, total lipid, and lipid class composition in eggs and embryos within ovaries of female yellowtail rockfish, *Sebastes flavidus*, and shortbelly rockfish, *Sebastes jordani*.

Materials and Methods

Female *S. flavidus* and *S. jordani* were obtained during January to March, the period of reproductive development spanning late vitellogenesis to parturition, at locations off the central California coast. Yellowtail rockfish were captured by hook-and-line at Cordell Bank, a marine bank 37 km west of Pt. Reyes, at depths ranging from 50 to 150 m. In 1994, shortbelly rockfish were collected by trawl in the proximity of three submarine canyons: Bodega Canyon (ca. 38°13'N 123°22'W), Pioneer Canyon (ca. 37°24'N 122°52'W), and Ascension Canyon (ca. 37°01'N 122°25'W), at 150 to 200 m depth. Fish were held on ice until examination. Morphometrics were recorded and ovaries were excised, weighed, and stored at -70°C. The stage of oocyte or embryo development was assessed by microscopy according to the classification scheme Yamada and Kusakari (1991) (Table 1).

Lipids were extracted from oocytes and embryos by the method of Bligh and Dyer (1959). Total lipids were quantified using thin layer chromatography with flame ionization detection (TLC-FID) by an Iatroscan TH-10 Mark III (MacFarlane et al., 1993). Lipid classes were separated on Chromarods S-III in a solvent bath of hexane:ethyl ether:formic acid at a ratio of 246:54:0.09. Quantification of separated peaks by TLC-FID was accomplished by comparing peak areas to external standard curves. Lipids were resolved into sterol/wax ester, triacylglycerol, nonesterified fatty acid, cholesterol, and polar lipid classes. Total protein was estimated by the Lowry method using a bovine serum albumin standard (Lowry et al., 1951). Analysis of variance (ANOVA) and linear regression were employed to assess variation in lipids and protein by embryo maturation stage (EMS), rockfish species, or population of shortbelly rockfish by SAS statistical software.

Table 1. Embryo maturation stages (EMS) in *Sebastes flavidus* and *Sebastes jordani*.

EMS	Description	EMS	Description
1	Late vitellogenic/ migratory nucleus oocyte	17	Optic vesicles
		18	Somite formation begins
2	Formation of germ disc	19	Finfold
3	2 - celled	20	Optic cups
4	4 - celled	21	Auditory placodes
5	8 - celled	22	Lens forms
6	16 - celled	23	Otoliths
7	32 - celled	24	Pectoral fins
8	64 - celled	25	Retinal pigmentation
9	Morula	26	Heart pumping
10	Early blastula	27	Lens transparent
11	Late blastula	28	Mouth and anus open
12	Beginning of epiboly	29	Peritoneum pigmented
13	Early gastrula	30	Yolk reduction
14	Late gastrula	31	Prehatching
15	Embryonic shield	32	Hatching
16	Head fold	33	Hatched, preborn larva

Results and Discussion

In yellowtail rockfish, there was a progressive decline in total lipid and protein during embryogenesis (Fig. 1). The concentration of total lipid decreased from a mean of 155.3 mg/g in unfertilized oocytes in the migratory nucleus stage (EMS 1) to an estimated concentration of 26.6 mg/g for fully-formed hatched larvae (EMS 33) at parturition. Although no pregnant females were caught with embryos at EMS 33, the goodness-of-fit of the linear regression of lipid concentration on embryo maturation stage for the stages collected (Table 2) suggested that calculation of total lipid at parturition was valid. Similarly, protein declined from 205.6 mg/g at EMS 1 to 36.0 mg/g at EMS 33 (Table 2).

Total lipid and protein concentrations declined linearly according to stage of development in embryos of shortbelly rockfish, also (Fig. 2). Highly significant linear relationships ($P < 0.0001$; Table 2) between total lipid or protein and EMS allowed estimation of nutrient concentrations at parturition. For all populations of shortbelly rockfish from the 3 submarine canyons, the estimated concentrations of total lipid and protein in larvae at parturition (EMS 33) were 21. 5 and 64.7 mg/g, respectively.

The concentrations and rates of metabolism of lipids and protein differed between yellowtail and shortbelly rockfish during their approximately 30 d gestation. The initial lipid and protein concentrations at the start of embryogenesis were greater in yellowtail rockfish than in shortbelly rockfish (t-test: $P < 0.05$ for protein; $P < 0.0001$ for total lipids); however, the rates of depletion were also greater in yellowtail rockfish ($P < 0.0005$). This resulted in similar concentrations of lipids at birth in the two species of *Sebastes*, but greater protein in shortbelly rockfish larvae.

Total lipid concentrations can be considered a component of condition assessment (Ferron and Leggett, 1994), and thus a measure of qualitative reproductive success, but not all types, or classes, of lipids are equal with respect to metabolic availability or energy yield. Therefore, fractionation of total lipids into classes representative of energy-yielding and structural functions provides greater knowledge of the amount of energy available to sustain growth and survival once the larvae are released into the environment.

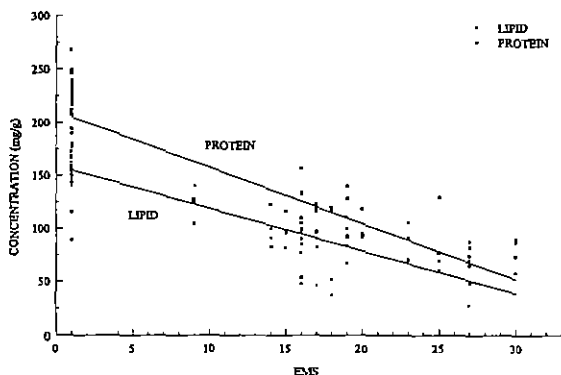

Figure 1. Total lipid and protein concentrations in oocytes and embryos in female yellowtail rockfish, *Sebastes flavidus*, from prefertilized oocytes (EMS 1) through the yolk reduction stage (EMS 30). See Table 1 for descriptions of EMS.

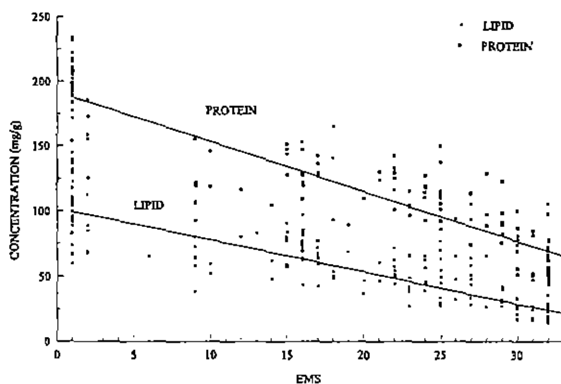

Figure 2. Total lipid and protein concentrations in oocytes and embryos in female shortbelly rockfish, *Sebastes jordani*, from prefertilized oocytes (EMS 1) through the hatched larvae stage (EMS 33). Data for shortbelly rockfish obtained from populations at all 3 submarine canyons are shown. See Table 1 for description of EMS.

Table 2. Linear regression parameter estimates for protein and lipid variables in rockfish embryos in relation to embryo maturation stage (EMS). Data are for yellowtail rockfish (*Sebastes. flavidus*) and three populations of shortbelly rockfish (*Sebastes jordani*) from Ascension, Pioneer, and Bodega Canyons.

Variable	r^2	Intercept±SE	P	Slope±SE	P
S. flavidus (n = 60)					
Total lipid	0.774	159.3 ± 4.4	<0.0001	-4.02 ± 0.29	<0.0001
Total protein	0.721	210.9 ± 6.7	<0.0001	-5.30 ± 0.44	<0.0001
S. jordani					
All Canyons (n = 182)					
Total lipid	0.771	103.7 ± 2.3	<0.0001	-2.49 ± 0.10	<0.0001
Total protein	0.793	193.1 ± 3.7	<0.0001	-3.89 ± 0.16	<0.0001
Esters	0.629	9.3 ± 0.3	<0.0001	-0.25 ± 0.01	<0.0001
TAG	0.743	50.4 ± 1.3	<0.0001	-1.32 ± 0.06	<0.0001
NEFA	0.013	0.0 ± 0.1	0.5937	0.01 ± 0.00	0.1224
CH	0.686	4.5 ± 0.1	<0.0001	-0.08 ± 0.00	<0.0001
PL	0.789	39.5 ± 0.7	<0.0001	-0.85 ± 0.03	<0.0001
Bodega Canyon (n = 19)					
Total lipid	0.751	84.9 ± 5.2	<0.0001	-1.67 ± 0.26	<0.0001
Total protein	0.716	190.1 ± 11.9	<0.0001	-3.85 ± 0.59	<0.0001
Esters	0.741	7.5 ± 0.5	<0.0001	-0.17 ± 0.02	<0.0001
TAG	0.708	39.3 ± 2.7	<0.0001	-0.86 ± 0.13	<0.0001
NEFA	ND	ND	ND	ND	ND
CH	0.475	3.4 ± 0.2	<0.0001	-0.04 ± 0.00	<0.001
PL	0.706	34.6 ± 1.9	<0.0001	-0.61 ± 0.10	<0.0001
Pioneer Canyon (n = 102)					
Total lipid	0.805	90.2 ± 2.8	<0.0001	-2.13 ± 0.11	<0.0001
Total protein	0.702	183.3 ± 6.7	<0.0001	-3.65 ± 0.25	<0.0001
Esters	0.730	7.6 ± 0.3	<0.0001	-0.20 ± 0.01	<0.0001
TAG	0.778	45.1 ± 1.7	<0.0001	-1.19 ± 0.06	<0.0001
NEFA	0.187	- 0.3 ± 0.1	0.010	0.02 ± 0.00	<0.0001
CH	0.709	4.3 ± 0.1	<0.0001	-0.08 ± 0.00	<0.001
PL	0.834	33.6 ± 0.8	<0.0001	-0.69 ± 0.03	<0.0001
Ascension Canyon (n = 61)					
Total lipid	0.541	107.5 ± 3.6	<0.0001	-2.03 ± 0.24	<0.0001
Total protein	0.671	193.0 ± 5.0	<0.0001	-3.12 ± 0.33	<0.0001
Esters	0.314	9.8 ± 0.6	<0.0001	-0.21 ± 0.04	<0.0001
TAG	0.469	51.8 ± 2.1	<0.0001	-1.02 ± 0.14	<0.0001
NEFA	0.011	0.2 ± 0.2	0.152	-0.01 ± 0.01	0.411
CH	0.445	4.6 ± 0.1	<0.0001	-0.07 ± 0.01	<0.0001
PL	0.589	41.1 ± 1.2	<0.0001	-0.72 ± 0.08	<0.0001

Lipid extracts from oocytes and embryos of shortbelly rockfish were separated into sterol/wax esters, triacylglycerols (TAG), nonesterified fatty acids (NEFA), cholesterol (CH), and polar lipids (PL). TAG and esters are energy-yielding lipids whereas CH and PL are considered to be primarily structural in purpose. PL consists of several groups of lipids including sphingomyelin and cerebrosides, but the great majority of the molecules in this class are phospholipids, the principal component of biological membranes. Although PL can be considered a class of structural lipids, phospholipids have been shown to be metabolized during embryo development in fish (Tocher et al., 1985).

All classes of lipids declined during embryogenesis in shortbelly rockfish (Fig. 3) except NEFA which were at very low concentrations and did not vary linearly with EMS (Table 2). TAG and PL were the most abundant lipid classes and declined the most during development, indicating both classes were significant sources of energy. Esters also declined significantly, but were at very low levels and not a major source of energy. CH was the most stable lipid during embryo development, but showed a slight but statistically significant decrease. The relatively stable concentrations of CH suggest its use as a normalizer of energy-yielding lipid content to correct for differences in size or quantity of embryonic tissue as has been employed previously for larvae of oviparous species (Fraser, 1989).

When lipid class concentrations were assessed in embryos of shortbelly rockfish from separate populations there were differences in the amounts and rates of depletion during intraovarian development (Table 2). Two-way ANOVA determined significant differences among populations at three submarine canyon areas located within about a 1° latitude span off the central California coast. All lipid classes, except NEFA varied significantly by EMS ($P < 0.0001$), population ($P < 0.0001$), and the interaction of population and EMS ($P \leq 0.05$).

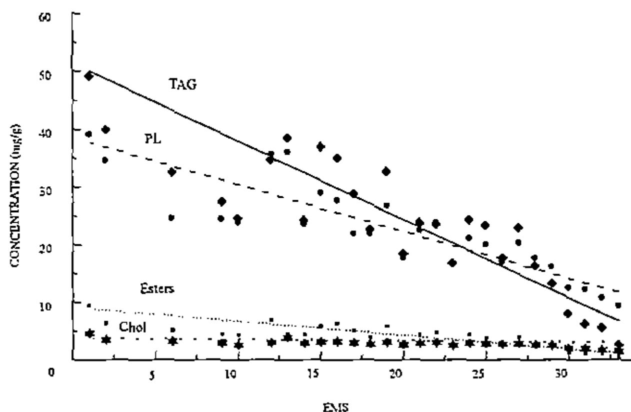

Figure 3. Changes in lipid classes during embryo development in shortbelly rockfish, *Sebastes jordani*. Data from representatives of populations at 3 submarine canyons. See Table 1 for description of EMS.

By solving regression equations for EMS 33 for lipid classes and protein in each population, estimated concentrations at birth can be obtained and compared among the populations (Table 3). These results indicate that there were differences in the condition of embryos at birth among the three populations, despite their relatively close geographical proximity. The estimated concentrations of protein and lipids in hatched, preborn larvae (EMS 33) in shortbelly rockfish at Ascension Canyon were significantly greater than those from the populations at Pioneer and Bodega Canyons. This suggests that the potential for survival during the critical period following birth is greater for the larvae from the Ascension Canyon population and may provide a greater contribution to the year-class of the species along the California coast.

Differences in energy-yielding lipid content may indicate relative survival potential in offspring from the various populations, especially during times of low biological productivity when food for pelagic larvae are scarce. Changes in environmental conditions, particularly those affecting the timing and intensity of upwelling, in the California Current ecosystem have been proposed as a cause of variable year-class strength in rockfishes (Moser and Boehlert, 1991).

The use of lipid and protein data as an indicator of condition at birth for viviparous rockfish larvae extends their application. Lipid class analysis has been employed for condition assessment in pelagic larval (Fraser, 1989; Håkanson, 1989; Lochmann et al., 1995) and juvenile stages (Suthers et al., 1992) of oviparous teleosts, and lipid class composition during embryonic development in Atlantic herring has been documented (Tocher et al., 1985). The assessment presented here provides a method to estimate energy reserves and biochemical composition of larvae at birth for viviparous species from field collections for the first time. Although knowledge of the length of gestation or of specific embryonic stages is not required, it may improve temporal resolution of the embryonic maturation stage scale and, thus, improve lipid and protein estimates at birth.

Table 3. Estimated concentrations of lipids and protein at birth for *Sebastes flavidus* and *Sebastes jordani*. *S. jordani* data are for all populations combined and for each of three populations at Bodega, Pioneer, and Ascension submarine canyons. Values presented as mean ± SD in mg/g wet weight.

	Sebastes flavidus	***Sebastes jordani***			
Variable		**All**	**Bodega**	**Pioneer**	**Ascension**
Protein	36.0 ± 9.6	64.7 ± 2.6 [1]	63.1 ± 9.7	62.8 ± 2.7	90.0 ± 7.4 [3]
Total lipid	26.6 ± 6.3	21.5 ± 1.7 [1]	29.8 ± 4.2 [2]	19.9 ± 1.2	40.5 ± 5.5 [3]
Esters	--	1.1 ± 0.2	1.9 ± 0.4 [2]	1.0 ± 0.1	2.9 ± 0.9 [3]
TAG	--	6.8 ± 1.0	10.9 ± 2.2 [2]	5.8 ± 0.7	18.1 ± 3.2 [3]
NEFA	--	0.3 ± 0.1	--	0.4 ± 0.0	0.0 ± 0.2 [3]
CH	--	1.9 ± 0.1	2.1 ± 0.2 [2]	1.7 ± 0.1	2.3 ± 0.2 [4]
PL	--	11.5 ± 0.6	14.5 ± 1.6 [2]	10.8 ± 0.4	17.3 ± 1.8 [3]

[1] Significantly different from *S. flavidus* (P < 0.0001)
[2] Greater than for *S. jordani* at Pioneer Canyon (P < 0.0001)
[3] Greater than for *S. jordani* at Bodega and Pioneer Canyons (P < 0.0001)
[4] Greater than for *S. jordani* at Bodega and Pioneer Canyons (P < 0.001)

References

Blaxter, JHS 1969 Development: eggs and larvae, p. 177-252. *In* WS Hoar and DJ Randall [ed.] Fish physiology. Vol. III. Academic Press, New York.

Blaxter, JHS and Hempel, G 1963 The influence of egg size on herring larvae (*Clupea harengus*). J. du Conseil International pour l'Exploration de la Mer 28: 211-240.

Bligh, EG and Dyer, G 1959 A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Buckley, LJ 1984 RNA-DNA ratio: an index of larval fish growth in the sea. Mar. Biol. 80: 291-298.

Clarke, ME, Calvi, C, Domeier, M, Edmonds, M, and Walsh, PJ 1992 Effect of nutrition and temperature on metabolic enzymes activities in larval and juvenile red drum, *Sciaenops ocellatus*, and lane snapper, *Lutjanus synagris*. Mar. Biol. 112: 31-36.

Ferron, A and Leggett, WC 1994 An appraisal of condition measures for fish larvae. Adv. Mar. Biol. 30: 217-303.

Fraser, AJ 1989 Triacylglycerol content as a condition index for fish, bivalve, and crustacean larvae. Can. J. Fish. Aquat. Sci. 46: 1868-1873.

Håkanson, JL 1989 Analysis of lipid components for determining the condition of anchovy larvae, *Engraulis mordax*. Mar. Biol. 102: 143-151.

Lochmann, SE, Maillet, GL, Frank, KT, and Taggart, CT 1995 Lipid class composition as a measure of nutritional condition in individual larval Atlantic cod (*Gadus morhua*). Can. J. Fish. Aquat. Sci. 52: 1294-1306.

Lowry, OH, Rosebrough, NJ, Farr, AL, and Randall, RJ 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

MacFarlane, RB, Norton, EC, and Bowers, MJ 1993 Lipid dynamics in relation to the annual reproductive cycle in yellowtail rockfish (*Sebastes flavidus*). Can. J. Fish. Aquat. Sci. 50: 391-401.

Moser, HG and Boehlert, GW 1991 Ecology of pelagic larvae and juveniles of the genus *Sebastes*. Environ. Biol. Fishes 30: 203-224.

Suthers, IM, Fraser, A, and Frank, KT 1992 Comparison of lipid, otolith and morphometric condition indices of pelagic juvenile cod *Gadus morhua* from the Canadian Atlantic. Mar. Ecol. Prog. Ser. 84: 31-40.

Theilacker, GH 1978 Effect of starvation on the histological and morphological characteristics of jack mackerel, *Trachurus symmetricus*, larvae. Fish. Bull. US 76: 403-414.

Tocher, DR, Fraser, AJ, Sargent, JR, and Gamble, JC 1985 Lipid class composition during embryonic and early larval development in Atlantic herring (*Clupea harengus*, L.). Lipids 20: 84-89.

Yamada, J and Kusakari, M 1991 Staging and the time course of embryonic development in kurosoi, *Sebastes schlegeli*. Environ. Biol. Fishes 30: 147-153.

EGG QUALITY OF GROUPER *EPINEPHELUS COIOIDES* FED DIFFERENT FATTY ACID SOURCES

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Abstract

Quality of eggs spawned by *Epinephelus coioides* fed fish bycatch (control), cod liver oil-enriched fish bycatch (TFC), and commercial HUFA-enriched fish bycatch (TFS) was monitored. Monthly egg production, spawning frequency, fertilization rate, egg viability, and hatching rates of the control were significantly higher compared to TFS. Egg production, spawning frequency, and hatching rate of TFC and TFS were not significantly different. Results suggests that varying the species of fish bycatch could provide the requirements of *E. coioides* broodstock so as to provide quality eggs.

Introduction

There is a high demand for groupers in Asia. However, production of cultured fishes is hampered by inadequate supply of seeds for stocking. Several workers have conducted research on seed production of various species of grouper. Some of these species are *Epinephelus tauvina* (Chen et al. 1977, Hussain et al. 1980, Chao and Lim 1991), *E. salmoides* (Kunvankij et al. 1986), *E. malabaricus* (Ruangpanit et al. 1986, Maneewong et al. 1986), *E. akaara* (for review, see Fukuhara 1989), *E. fuscoguttatus* (Lim et al. 1990, Chao and Lim 1991), and *E. suillus* (Doi et al. 1991). Despite advances in technology, these reports have shown low or inconsistent larval survival.

Toledo et al. (1993) have shown that the grouper *Epinephelus suillus* (=*E. coioides*) can spontaneously spawn year-round in tanks. Despite this, varying egg quality may be one of the limiting factors for successful mass production of fry (Kjorsvik et al. 1990). It may cause changes in recruitment and may decrease the survival potential of larvae. Supplementing the fat intake of the broodstock, therefore, could help in solving this problem (Dhert et al. 1991). Thus, this study was conducted in an attempt to improve the quality of spawned eggs by adding different sources of fat to the feed of grouper broodstock.

Materials and methods

Grouper (*E. coioides*) broodstock (females: 2.5-6.5 kg; males: 8.3-11.0 kg body weight, BW) were stocked in three 50-t concrete tanks, each containing 1 male and 3 females. A flow-through water system with aeration was used. The tanks were shaded by a black plastic straw mat to minimized growth of algae and diatoms. From 1 November 1991 to 28 October 1992, the fish were fed fish bycatch (trash fish) only (Control), fish bycatch enriched with cod liver oil (TFC), and fish bycatch enriched with a commercial highly unsaturated fatty acid (SELCO) (TFS). Broodstock were fed to satiation every other day.

Naturally spawned eggs were collected the following morning after spawning. This was done by shutting off the aeration for about 15 min. Floating eggs were seined with a fine mesh net while sinking eggs were collected by draining the tank. Seined and drained eggs were stocked separately in incubation tanks filled with 200 l filtered seawater. Parameters such as total eggs spawned, fertilization rate, percentage of viable eggs, hatching rate, percentage of normal larvae, and

survival of larvae without feeding were determined based on some criteria cited by Kjorsvik et al. (1990).

The crude protein and crude fat of floating and sinking spawned eggs and fish bycatch enriched with different sources of fatty acids were analyzed. Crude protein was determined using Semi-automated Method utilizing a Kjeltec Auto analyzer while the Soxtec Method was used for crude fat.

Water temperature and salinity were monitored daily at about 15.00 h. Monthly mean water temperature and salinity ranges were 26.8-29.6 °C and 30.6-34.1 ppt, respectively.

Analysis of variance on a randomized complete block design was used for statistical analysis. Log and square root transformations of egg count and spawning frequency values, respectively, were made before analysis. All values expressed in percent were arcsin transformed before analysis.

Results

Spawning commenced between the last quarter and new moon of the lunar cycle and in most cases 1-3 days after the last quarter moon. The total egg production from January to October 1992 in the control fish was about 77.9 million eggs, 40.0 million in TFC fish, and 36.4 million in TFS (Fig. 1). Range of monthly egg production on a per weight basis in the control was from 0.18 to 0.45 million eggs/kg BW, while in TFC and TFS the range were 0.06-0.36 and 0.01-0.41 million eggs/kg BW, respectively. Analysis of variance showed that mean monthly egg production in the control (0.34 million eggs/kg BW) and TFC (0.21 million eggs/kg BW) were not significantly different while both are significantly different ($P<0.05$) from that of TFS (0.15 million eggs/kg BW). Mean monthly spawning frequency was highest in the control (6.0 days, Table 1) but was not significantly different from TFC fish (5.2 days). TFS fish significantly gave the lowest monthly spawning frequency (3.4 days).

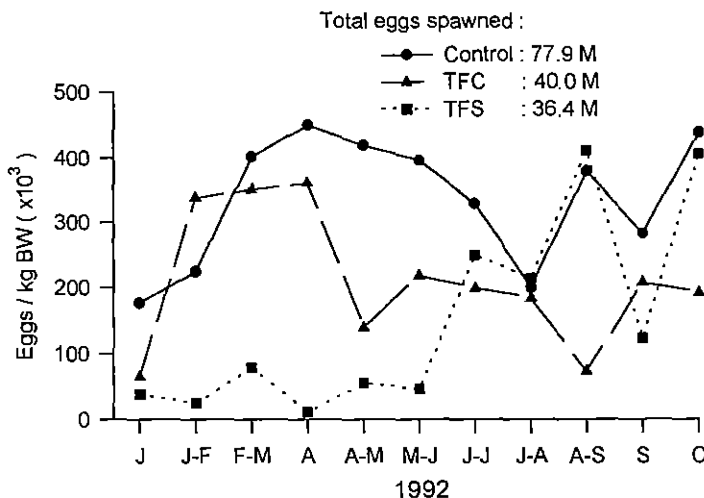

Figure 1. Egg production of grouper broodstock fed different sources of fatty acids. Control - fish bycatch only; TFC - cod liver oil-enriched fish bycatch; TFS - SELCO -enriched fish bycatch.

Fertilization rate, egg viability, and hatching rate (Table 1) showed significant differences ($P<0.01$) among the three groups. The control gave the highest values but hatching rate was not different to that of TFC. TFS values were lowest but the mean fertilization rate was not different

to that of TFC. No significant differences were detected in the oil globule (0.18-0.21 mm) and egg (0.78-0.89 mm) diameters. Moreover, percentage of normal larvae among the treatments were not significantly different (Table 1).

Table 1. Spawning frequency, fertilization rate, hatching rate, and percentage of normal larvae of grouper broodstock fed different sources of fatty acid. Control - fish bycatch only; TFC - cod liver oil-enriched fish bycatch; TFS - SELCO-enriched fish bycatch. Values are expressed as mean ± SEM. Values with the same superscript in a column are not significantly different ($P < 0.05$).

Diet	Spawning frequency (days)	Fertilization Rate (%)	Egg Viability (%)	Hatching Rate (%)	% Normal larvae
Control	6.0±0.6[a]	70.94±3.45[a]	32.28±6.98[a]	52.80±5.04[a]	79.39± 2.66[a]
TFC	5.2±0.1[a]	48.12±5.44[b]	24.37±5.31[a]	34.55±3.46[b]	81.37± 3.20[a]
FS	3.4±0.7[b]	41.99±6.49[b]	12.43±4.96[b]	23.85±5.90[c]	61.69±11.48[a]

The survival of unfed newly-hatched larvae from every spawning day in the different treatments was monitored during the 24 July-1 August, 24 August-2 September, and 21-29 September 1992 spawning months. Larvae from the TFC group survived until the fifth day after hatching while those from control and TFS groups lasted only until the third day. However, no significant difference was observed among treatments.

Crude protein (46.00-55.20%) and fat (16.79-24.65%) of floating and sinking eggs collected in February to March 1992, and August to September 1992 were similar in all treatments (Table 2).

Table 2. Mean crude protein and crude fat of floating and sinking eggs of grouper broodstock fed different sources of fatty acid spawned from 27 February to 9 March 1992 and 24 August to 2 September 1992. Control - fish bycatch only; TFC - cod liver oil-enriched fish bycatch; TFS - SELCO-enriched fish bycatch.

	Floating eggs		Sinking eggs	
Diet	Crude Protein	Crude Fat	Crude Protein	Crude Fat
Control	53.22	18.76	50.86	23.02
TFC	55.20*	16.79	47.76	22.56
TFS	50.00	23.24	46.00	24.65

*Value of eggs spawned from 27 February to 9 March 1992 only.

The species of fish bycatch given to the broodstock depended on the available fish in the market. These composed of *Selaroides* sp., *Nemipterus* sp., *Rastrelliger* sp., and *Decapterus* sp. (Table 3). Results showed that crude protein decreased ($P<0.01$) when cod liver oil or SELCO (59.51-71.20%) was added in all the fish bycatch species given while crude fat increased (12.62-23.15%).

Discussion

In this study, spawning of *E. coioides* broodstock was similar to the observations of Toledo et al. (1993). In most cases, spawning commenced 1-3 days after the last quarter moon phase.

The results of this study indicate that supplementation of cod liver oil and SELCO in the fish bycatch of *E. coioides* broodstock did not improve egg production and other parameters determined. In some cases, these fatty acid sources even gave negative results. Dhert et al. (1991) showed that *E. tauvina* broodstock given fish bycatch injected the emulsified enrichment diet Marila diet significantly increased oil globule diameter, total lipids, eicosapentaenoic acid, docosahexaenoic acid, and larval survival at day 7. However, the species of fish bycatch given was not identified. Watanabe (1985) indicated that there is a need for a careful balance of essential fatty acid (EFA) in the diet of broodstock. It may be possible that feeding varied fish bycatch to *E. coioides* broodstock was able to provide the EFA requirement while addition of CLO and SELCO affected the EFA levels.

Table 3. Mean crude protein and crude fat of the different species of fish bycatch fed to the grouper broodstock. Means with the same superscript in a column of a species are not significantly different ($P < 0.01$).

Species	Crude Protein(%)	Crude Fat(%)
Selaroides sp.		
Control	67.57^{a}	13.24^{c}
TFC	59.96^{c}	22.48^{a}
TFS	65.20^{b}	19.60^{b}
Decapterus sp.		
Control	65.95^{a}	19.11^{c}
TFC	63.00^{c}	23.15^{a}
TFS	64.40^{b}	21.50^{b}
Nemipterus sp.		
Control	67.08^{a}	11.38^{b}
TFC	63.52^{b}	18.69^{a}
TFS	59.51^{c}	19.33^{a}
Rastrilleger sp.		
Control	74.86^{a}	8.68^{c}
TFC	69.32^{c}	17.23^{a}
TFS	71.20^{b}	12.62^{b}

Levels of crude protein and crude fat of floating and sinking eggs were similar in all the treatments. This indicates that proximate analysis could not show differences of spawned eggs. Determination of the fatty acid profile would have been the more appropriate technique to actually see differences.

Nevertheless, our results have shown that giving varied species of fish bycatch to grouper broodstock is able to provide the dietary requirement which could give quality eggs. It may also be worthwhile conditioning the fish to feed on artificial diet so that the actual lipid source and requirements for improving egg quality could be determined.

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References

Dhert P, LC Lim, P Lavens, TM Chou, R Chou, P Sorgeloos 1991 Effect of dietary fatty acids on egg quality and larviculture success of the greasy grouper (*Epinephelus tauvina*, F.): Preliminary results. In Lavens P, P Sorgeloos, E Jaspers,. F Ollevier (Eds.) Larvi '91-Fish and Crustacean larviculture Symposium. European Aquaculture Society, Spl. Publ. 15, Gent Belgium: 58-62.

Kjorsvik E, A Mangor-Jensen, I Holmefjord 1990 Egg quality in fishes. Adv. in Mar. Biol. 26: 71-113.

Maneewong S, P Akkayanont, J Pongmaneerat, M Iizawa 1986 Larval rearing and development of grouper, *Epinephelus malabaricus* (Bloch and Schneider). Report of the Thailand and Japan Joint Coastal Aquaculture Research Project (April 1984-January 1986) No. 2: 39-52.

Ruangpanit N, S Maneewong, T Tattanon, P Kraisingdecha, P Akkayanont, S Rojanapitayagul 1986 Preliminary study on rearing fry of grouper, *Epinephelus malabaricus*. Report of the Thailand and Japan Joint Coastal Aquaculture Research Project (April 1984-January 1986) No. 2: 35-38.

Kunvankij P, LB Tiro, BP Pudadera, IO Potestas 1986 Induced spawning and larval rearing of grouper (*Epinephelus salmoides* Maxwell), p. 663-666. In: Maclean JL., LB Dizon, LV Hosillos (Eds.) The First Asian Fisheries Forum. Asian Fisheries Society, Manila, Philippines.

Hussain NA, M Higuchi 1980 Larval rearing and development of the brown spotted grouper, *Epinephelus tauvina* (Forskal). Aquaculture 19: 339-350.

Doi M, MM Nawi, NR Nik Lah, Z Talib 1991 Artificial propagation of the grouper, *Epinephelus suillus*, at the Marine Finfish Hatchery in Tanjong Demong, Terengannu, Malaysia. Dept. of Fisheries, Ministry of Agriculture, Malaysia. Bilangan 167. 41 p.

Fukuhara O 1989 A review of the culture of grouper in Japan. Bull. Nansei Reg. Fish. Res. Lab. No. 22: 47-56.

Chen FY, M Chow, TM Chao, R Lim 1977 Artificial spawning and larval rearing of the grouper, *Epinephelus tauvina* (Forskal) in Singapore. Singapore J. Prim. Ind. 5: 1-21.

Chao TM, LC Lim 1991 Recent developments in the breeding of grouper (*Epinephelus* spp.) in Singapore. Singapore J. Prim. Ind. 19: 78-93.

Lim LC, TM Chao, LT Khoo 1990 Observations on the breeding of brown-marbled grouper, *Epinephelus fuscoguttatus* (Forskal). Singapore J. Prim. Ind. 18: 66-84.

Toledo JD, A Nagai, D Javellana 1993 Successive spawning of grouper, *Epinephelus suillus* (Valenciennes), in a tank and a floating net cage. Aquaculture 115: 361-367.

Watanabe T 1985 Importance of the study of broodstock nutrition for further development of aquaculture. In: Cowey CB, AM Mackie, SG Bell (Eds.) Nutrition and Feeding in Fish. Academic Press, London: 395-414.

EGG SIZE DETERMINES OFFSPRING SIZE IN NEOTROPICAL CICHLID FISHES

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Abstract

We report a positive correlation between egg and hatchling size both across a range of twenty-one neotropical cichlid species and within individual clutches in two species. We also reveal a positive correlation between egg size and the yolk size of hatchlings. Because maximization of reproductive success involves a spectrum of trade-off decisions, mean egg size and degree of deviation from this mean within a clutch may have far reaching consequences for other aspects of parental investment and for the life-history parameters of the hatchlings themselves.

Introduction

Within the family Cichlidae, the size of eggs varies tremendously across species (Breder and Rosen, 1966; Fryer and Iles, 1972; Coleman, 1991; Coleman, in prep). Sizes range from the minute eggs (0.9mm in diameter) of rams (Microgeophagus ramirezi) to the massive eggs (4.5mm in diameter) of some of the African mouthbrooding cichlids such as Cyphotilapia frontosa. At the extremes, larger eggs clearly produce larger offspring; however, this is not immediately apparent within smaller groupings in the family. For example, within the Neotropical cichlids, some species have an egg diameter of 1.6 mm, others 1.4mm. Does this relatively small difference in egg size actually translate into a consistent difference in the size of the hatchlings? Furthermore, does the variation in egg size within a single laying or clutch account for differences in hatchling size?

Myriad selection pressures could act on egg size in cichlids. In addition to the tradeoff between egg number and size of egg (Smith and Fretwell, 1974), size might determine the time to hatch; the size, health or capabilities of the hatchling; and the surface area to volume ratio of the egg which is important in gas exchange. These factors in turn may interact with the duration and form of parental care. Although parental care is universal in cichlids, its precise nature varies widely across taxa from simple fanning and guarding to prolonged parental care

and mouthbrooding and may involve either one or both parents (Keenleyside, 1991). Understanding the relationship between size of egg and hatchling is a first step toward deciphering the evolution of egg size in general.

A larger egg may not translate into a larger hatchling in several ways. For example, our measure of egg size may not be a true representation of the size of an egg. An ideal measure of egg size might involve a complete biochemical analysis of the contents of an egg, reporting protein, lipid and carbohydrate content (e.g., Kamler, 1992); however, this is not practical for most studies. Indeed, for cichlids, even weighing individual eggs or measuring their volume is difficult because of their small size.

The most practical approach to represent egg size is a linear measurement of egg diameter: it is quickly and easily done under a microscope or in a pinch (e.g., in the field) using a magnifying glass and a ruler. However, it has a few pitfalls. Cichlid eggs are not spherical, but are better described as prolate spheroids (roughly the shape of a cantaloupe), though exceptions exist. The eggs of African mouthbrooders in particular tend toward the shape of a chicken egg in some cases; others have a distinctive pear shape (pers. obs.). The eggs of some pike cichlids (genus Crenicichla) are markedly more elongate than other cichlid eggs and the eggs of oscars (Astronotus ocellatus) are much more spherical (pers. obs.). To deal with this variation, Coleman (1991) proposed using the "effective diameter" of nonspherical eggs as a means for comparison. The effective diameter is the diameter the egg would have if its contents were reshaped into a perfect sphere. In the case of a prolate spheroid, the effective diameter is the cube root of the major axis multiplied by the square of the minor axis (i.e., cube root of length times width times width). Throughout this paper we use effective diameter (d_e) as our measure of egg size.

Egg size may not translate into a larger hatchling if the quantity we measure is not a good measure of what is important in producing a hatchling. For example, larger eggs may simply be filled with more water than smaller eggs, and size differences may solely be accounted for by water content. In such a case, the difference in egg size may be insignificant to the resulting hatchling. This is particularly plausible because fish eggs are hydrated and expand to some extent when they leave the female (Kamler, 1992).

To address these questions, we investigated the relationship between egg size and hatchling size in twenty-one species of Neotropical cichlids. We also examined individual eggs and hatchlings from within single clutches to look for intra-clutch variation.

Methods

Interspecific comparisons.-- The twenty-one species examined were Aequidens pulcher, Cichlasoma bimaculatum, C. dimerus, C. festae, C. pusillum, 'C.' (Amphilophus) citrinellum, 'C.' (Am.) longimanus, 'C.' (Nandopsis) managuense, 'C.' (Archocentrus) centrarchus, 'C.' (Ar.) nigrofasciatum, 'C.' (Ar.) septemfasciatum, 'C.' (Ar.) spilurum, 'C.' (Theraps) bifasciatum, 'C.' (Th.) nicaraguense, 'C.' (Th.) sieboldii, 'C.' (Th.) synspillum, 'C.' (Th.) tuba, Crenicichla lepidota, Herotilapia multispinosa, Microgeophagus altispinosa and Neetroplus nematopus.

The fish were maintained in the laboratory using a typical freshwater aquarium setup, although the details varied and are of no direct relevance. A typical setup consisted of a glass aquarium of 38 to 280 litres, gravel, sponge filter, heater, and either pieces of slate, flowerpots or broken flowerpots. All species examined were substrate spawners (Loiselle, 1994) laying adhesive eggs, though differing in the precise choice of spawning site. Some species selected exposed surfaces (e.g., C. bimaculatum, C. pusillum) while others chose a cave-like spawning site, provided by the inside surface of a bottomless flowerpot laying on its side (e.g., 'C.'

nigrofasciatum, Cr. lepidota). Water temperatures were maintained between 24 and 28°C and the light cycle was 12D/12L. Fish were fed twice daily on Purina trout chow and Tetramin cichlid flakes with weekly supplements of live brine shrimp.

Fish were observed for the presence of eggs or hatchlings at least once daily. When eggs were first seen, a sample of 30 to 50 were scraped from the substrate with a blunt metallic spatula. Other eggs were left to hatch. The rubbery texture of cichlid eggs ensures that brief handling has no effect on egg size nor on egg hatchability. Eggs were then either measured immediately or stored in 70% isopropyl alcohol. Storage in isopropyl alcohol does not significantly alter egg size. To calculate effective diameter, we measured the length and width of 20 eggs using a Wild dissecting scope fitted with an ocular micrometer, calibrated to 1/100th of a millimeter. The 20 values were averaged to produce a mean egg size for the species.

On the day of hatching, hatchlings were collected with either an eyedropper or a turkey baster. Because live hatchlings are difficult to measure accurately, they were immediately preserved in 4% formalin and measured several days later. Three parameters were measured with each hatchling lying on its side: the total length of the hatchling, and the length and width of the yolk sac. Typically, 20 hatchlings were measured to obtain a mean hatchling value for the species, though in a few cases, particularly when hatchlings were rare, fewer than 20 were used ('C.' tuba 3; 'C.' sieboldii 12; 'C.' synspillum 19, M. altispinosa 11). We used only a single spawning for each species.

To evaluate the range of errors associated with the data-collection technique, we also took daily samples of hatchlings up to the stage of free-swimming from two species, 'C.' citrinellum and 'C.' synspillum.

To test whether egg size explains offspring size, we regressed mean hatchling length on mean egg size, and we regressed mean yolk diameter on mean egg size. If larger eggs are simply filled with more water, we would not expect significant positive relationships in either regression.

Intraspecific comparisons.-- To assess variation within a clutch in size of egg and offspring, we obtained individual hatchlings from eggs of known size using single clutches of 'C.' nigrofasciatum and H. multispinosa. Eggs were scraped off the laying site as described above and their size examined in a petri dish. To maximize the range of egg sizes used, extreme sizes were selected as well as eggs of more intermediate size. Each egg was measured under the microscope, then placed in a numbered hatching cup in the hatching aquarium. As each egg hatched several days later, the hatchling was removed and placed in a numbered vial of 4% formalin. Eggs that did not hatch were dropped from subsequent analysis. The hatchlings were measured as above and similar regressions performed. Coefficient of variation (CV) was calculated as standard deviation (SD) times 100 divided by the mean (Sokal and Rohlf, 1981).

Hatching cups were made from the bottom portion of a plastic specimen vial siliconed to a small square of glass. Each cup has a diameter of 2.0 cm and a height of 1.5 cm. The glass serves to make the cup sink. The cups were placed in a half-filled 38 l aquarium, with a heater and airstone. This hatching-cup design proved successful in other experiments because it allows enough circulation of water over the eggs, but does not cause them to be blown out of the cups.

Results

Interspecific comparisons.-- Mean hatchling length was positively correlated with mean egg diameter in the twenty-one species examined (Fig. 1; $r = 0.87$; $df = 19$; $P << 0.01$). The figure also illustrates the length of hatchlings of 'C.' synspillum on days one through six following

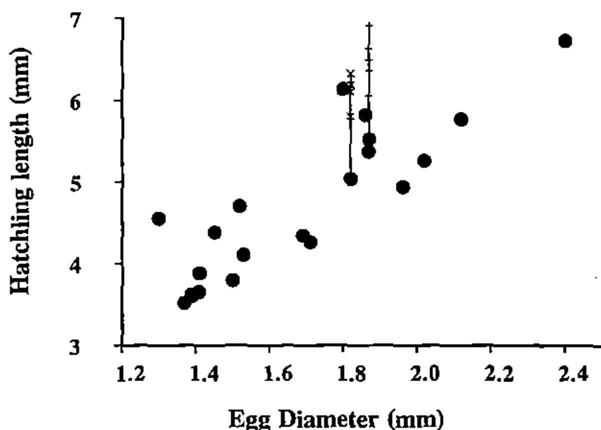

Figure 1. Mean Hatchling length (HL) as a function of mean effective egg diameter (d_e) for twenty-one species of cichlids (HL = 3.07 x d_e - 0.33 mm). The vertical bars show the increasing length of hatchlings from a single brood on subsequent days. The bar on the left is for 'C.' citrinellum, while the one on the right is for 'C.' synspillum.

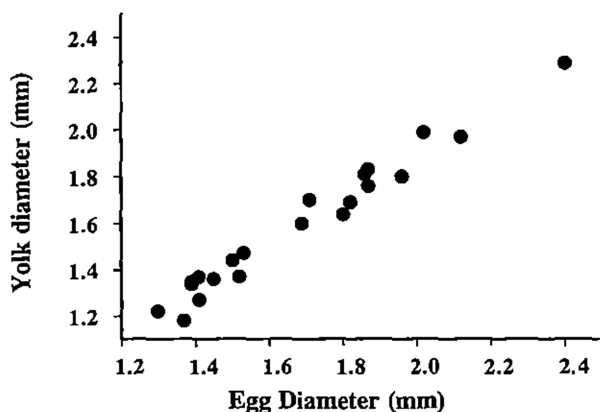

Figure 2. Mean Yolk diameter (y_e) of hatchlings as a function of mean effective egg diameter (d_e) for twenty-one species of Neotropical cichlids (y_e = 0.99 x d_e - 0.08 mm).

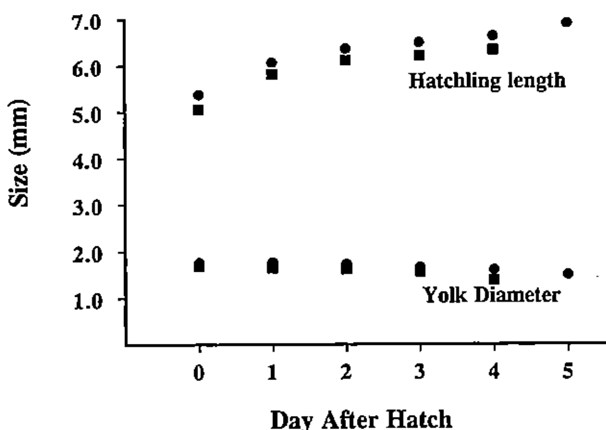

Figure 3. The change in hatchling length and yolk diameter on the days after hatching. Circles represent '<u>C</u>.' <u>synspillum</u>, squares represent '<u>C</u>.' <u>citrinellum</u>.

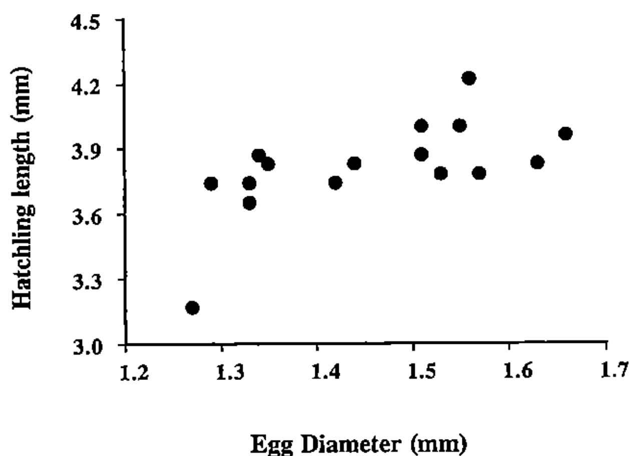

Figure 4. Hatchling length (HL) of individual hatchlings as a function of effective egg diameter (d_e) within a single clutch of rainbow cichlids ($HL = 1.09 \times d_e + 2.23$mm). A plot of yolk diameter (y_e) on effective egg diameter (d_e) looks similar ($y_e = 0.97 \times d_e - 0.05$mm).

hatching at 25.0 °C and 'C.' citrinellum on days one through five following hatching at 26.7 °C. This indicates the rate of change of hatchling size through time. Most of the eggs fall within the range of effective diameters of 1.3 to 1.9 mm. The extreme point at 2.4 mm is 'C.' tuba, the only known cichlasomine with such a large egg.

A significant positive correlation between the means for hatchling yolk diameter and egg diameter in these species was also uncovered (Fig. 2; $r = 0.98$; $df = 19$; $P << 0.01$). In the days after hatching, the yolk diameter continues to decline as the hatchling length increases (Fig. 3).

Intraclutch comparisons.-- Within individual clutches, we found a positive correlation between both hatchling and egg size and yolk diameter and egg size. For rainbow cichlids (H. multispinosa), hatchling length increased with egg diameter (Fig. 4; $r = 0.62$; $df = 14$; $P < 0.05$), and yolk diameter increased with egg diameter ($r = 0.93$; $df = 14$; $P << 0.01$). Removing the possible outlier at egg size of 1.27 (Fig. 4) did not significantly change either result. The coefficient of variation for egg diameter, yolk diameter and hatchling length were 8.6%, 9.4% and 5.8% respectively.

For convict cichlids ('C.' nigrofasciatum), the results were similar for hatchling length ($r = 0.63$; $df = 12$; $P < 0.05$) and yolk diameter ($r = 0.90$; $df = 12$; $P << 0.01$). The coefficient of variation for egg diameter, yolk diameter and hatchling length were 5.5%, 6.1% and 2.9% respectively.

Discussion

Eggs are one of the most quantifiable of life history stages of an organism. This is particularly true for fish, which exhibit indeterminate growth, making it difficult to even state such other life history parameters as the size of an adult for many species. Although there is a large literature reporting egg size values for individual species, and to a lesser extent, information on hatchling size, there have been surprisingly few attempts to pull this data together in a life-history context (e.g., Moser et al., 1984; Kamler, 1992). Likely this is because of the relatively small size of most fish eggs, often 1 to 2 mm in diameter. The salmonid egg literature is exceptional because of the tremendous economic importance of salmonid egg research (e.g., survival in the wild, captive propagation), plus the large size of salmonid eggs, often 6-10 mm in diameter, which allows researchers to conduct detailed investigations. For example, it is possible to weigh with some accuracy individual salmonid eggs. Cichlid eggs are too small to be weighed, but the linear size of a cichlid egg is an important piece of information that can be used in understanding cichlid reproductive strategies and life history patterns.

Our results indicate that a considerable source of hatchling-size variation derives from differences in egg sizes. This relationship holds both across species of Neotropical cichlids and within single clutches. Across species, differences in egg size accounted for 79% of the variation in hatchling total length, and 97% of the variation in the size of the yolk of hatchlings. We have insufficient information at this point about the life history of each species to attribute any specific relevance to the fry size of a particular species. The results certainly suggest that we should pursue this question. For example, the strikingly large egg and fry of 'C.' tuba beg for an explanation.

Within a single clutch of rainbow cichlids, egg size accounted for 38% of the variation in hatchling total length, and 87% of the variation in yolk size of hatchlings. Similarly, for a clutch of convict cichlids we found 40% and 81% respectively. Our intraclutch results differ from a previous report by Lagomarsino et al. (1988) who detected only a weak correlation between egg size and hatchling length in the midas cichlid, 'C.' citrinellum. They argued that

their correlation was so weak as to be biologically insignificant. We suspect three possible reasons why Lagomarsino et al. (1988) found only a weak correlation. First, it appears, though it is not explicitly stated, that they measured live fry, not preserved ones. Second, they measured the hatchlings at free-swimming, when the yolk sac was completely absorbed, several days later in development than our study. Third, they used random samples of eggs from each of the broods they examined whereas we specifically selected a range of eggs of different sizes. If we examine the coeffecient of variation in egg size for each of their broods using the data they provide in Table 1, the CVs are in almost all cases comparable to the CVs we used (range = 6.9 to 19.3, mean = 11.0). As they point out, the CVs they found for fry length were much smaller (range = 1.3 to 6.1, mean = 2.7). The question remains whether this lack of variation in free-swimming hatchling size was the result of measuring live hatchlings or if the variation they found in size of eggs truly disappeared after the eggs hatched.

We did not attempt to follow hatchlings beyond the point of hatching to determine whether these differences in hatchling size within clutches persist. Once the hatchlings reach the free-swimming stage, a few days after hatching, tracking individuals is technically impossible. They are too small to mark individually in a way that would not severely compromise their condition. However, within a matter of weeks after hatching dramatic differences appear in the size of offspring from a single clutch of cichlids. For example, Valerio and Barlow (1986) found that at 43 days of age, the largest fish were able to prey upon their smaller siblings. The coefficient of variation of fry size in their study increased from 2.5% at one week after hatching to 23% on day 62. After this it decreased to 5%, likely because the smaller animals were being eaten by the larger. Part of the variation in fry size doubtlessly arises from differential access to food, the larger individuals excluding or at least restricting access to food by the smaller individuals. The phenomenon of 'growth depensation', namely that through time the largest fish in a cohort get larger and the smaller fish lag further behind, is widespread in fishes (Brown, 1957; Brett, 1979).

An intriguing question remains: does some tiny difference in egg size give certain individuals a slight size advantage at hatching, one that persists and is magnified throughout the individual's life? Or, do these initial differences disappear and other forces generate the differential growth rates? If the initial size, then a substantial portion of an offspring's life-history may be under maternal control. For example, Francis and Barlow (1993) have recently found evidence that sex determination in these cichlids is influenced by their social environment, not sex chromosomes: the largest individuals in a group becoming male and the smaller ones becoming female. As Barlow (pers. comm.) has suggested, if the offspring from several clutches mix as they mature, a female could conceivably alter the sex ratio of her offspring by producing larger or smaller eggs. This might best be examined in haremic cichlids, such as Apistogramma, where there is extreme sexual dimorphism in size (Barlow 1991). A female could make a few extra large eggs to become males and many more smaller ones to become females.

The salmonid literature is ambiguous about the persistence of initial size differences. Most studies suggest that initial differences in the size of hatchlings from small and large eggs disappear through time (Springate and Bromage, 1985). However, the goal of this literature is often different than what we seek in this paper. The salmonid research is aimed at asking whether the initial differences persist despite the efforts of aquaculturists to compensate for the slow start of small eggs, i.e., they want to know if small eggs are bad for producing large salmon. This is quite different than asking whether the initial differences between hatchlings persist, and have effects, under natural conditions.

The positive association between the yolk size of hatchlings and egg size indicates a metabolic cost to producing larger eggs. Larger eggs are not larger because they are filled with more water, but because they contain more of the materials necessary to produce a larger hatchling,

the effects of which continue after hatching. Therefore from a parental investment perspective, making a large egg costs a female more per egg than making a small egg. This may not always be the case, particularly if we compare across divergent fish taxa (Kamler 1992). For example, many marine fish lay buoyant eggs which float in the plankton (Russell, 1976). To compare these eggs against cichlid eggs, which are slightly negatively buoyant, we would have to correct for the different densities. In such a case, yolk content is probably the most meaningful measure of investment, though it may be impractical to assess. For cichlids, our results show egg size to be a strong predictor of hatchling yolk size so we can assume that the density of eggs is roughly similar across the range of species studied.

Larger eggs entail a reduction in the number of eggs that can be produced (Smith and Fretwell, 1974; Coleman, in prep.). Therefore, we postulate larger size must confer a compensatory advantage, such as the ability to escape predators, to exploit an inhospitable environment or to consume a broader array of foods. No studies to date have examined this question at such a fine level of variation in the Cichlidae.

The daily samples of 'C.' citrinellum and 'C.' synspillum illustrate the magnitude of errors inherent in the sampling design. The eggs were checked at least daily for signs of hatching, making the maximum error in finding hatchlings one day. We see that one day's growth would not significantly alter the overall picture, but it may explain some of the remaining variation in hatchling size. Moreover, the data illustrate that a 'C.' citrinellum or a 'C.' synspillum hatchling will acheive in five or six days the size of a 'C.' tuba hatchling on its first day. This suggests that the benefits of large fry size in 'C.' tuba must outweigh the costs of producing large eggs in a relatively short time.

In fishes that provide parental care such as cichlids, minimizing egg-size variation within clutches may be advantageous. If larger fry have also reached a more advanced developmental stage, a clutch containing a range of sizes, and therefore developmental stages, will be difficult to care for. Inevitably, some fry will not be receiving care appropriate to their stage of development.

Parental cichlids perform distinct acts that change as the offspring develop. For instance, most substrate-spawning species fan the eggs. At hatch, many species immediately move the hatchlings to a pit in the substrate. Once the fry become free-swimming, the fry move about as a school and the parents guard the school. Field observations of some species reveal these schools roving over several meters, up and down a river (pers. obs.). Whether the parents direct the school or if they go where the fry go is not always clear. In either case, if some eggs were still unhatched when other fry started moving, that would decrease the parents' abilities to care for all the fry effectively.

Acknowledgements

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References

Barlow GW (1991) Mating systems among cichlid fishes, p. 173-190; in: Cichlid Fishes: Behaviour, ecology and evolution. Keenleyside MHA (ed) Chapman and Hall, New York, New York.

Breder CM Jr., and Rosen DE (1966) Modes of Reproduction in Fishes. TFH Publications, Neptune City, New Jersey.

Brett JR (1979) Environmental factors and growth, p. 599-675; in: Fish physiology, volume 8, Bioenergetics and growth. Hoar WS, Randall DJ and Brett JR (eds) Academic Press, New York, New York.

Brown ME (1957) Experimental studies of growth, p. 361-400; in: Physiology of fishes, volume 1. Brown ME (ed) Academic Press, New York, New York.

Coleman RM (1991) Measuring parental investment in nonspherical eggs. Copeia 1991:1092-1098.

Francis RC, and Barlow GW (1993) Social control of primary sex differentiation in the midas cichlid. Proc. Natl. Acad. Sci. USA 90:10673-10675.

Fryer G and Iles TD (1972) The cichlid fishes of the Great Lakes of Africa. TFH Publications, Neptune City, New Jersey.

Kamler E (1992) Early life history of fish: an energetics approach. Chapman and Hall, New York, New York.

Keenleyside MHA (1991) Parental care, p. 191-208; in: Cichlid Fishes: behaviour, ecology and evolution. Keenleyside MHA (ed) Chapman and Hall, New York, New York.

Lagomarsino I, Francis RC and Barlow GW (1988) The lack of correlation between size of egg and size of hatchling in the midas cichlid, Cichlasoma citrinellum. Copeia 1988:1086-1089.

Loiselle PV (1994) The cichlid aquarium (revised and expanded ed.) Tetra-Press, Melle, Germany.

Moser HG, Richards WJ, Cohen DM, Fahay MP, Kendall AW Jr. and Richardson SL (eds) (1984) Ontogeny and systematics of fishes. Am. Soc. Ich. Herp. Spec. Publ. 1.

Russell FS (1976) The eggs and planktonic stages of British marine fishes. Academic Press, London.

Smith CC and Fretwell SD (1974) The optimal balance between size and number of offspring. Am. Nat. 108:499-506.

Springate JRC and Bromage NR (1985) Effects of egg size on early growth and survival in rainbow trout (Salmo gairdneri Richardson). Aquaculture 47:163-172.

Sokal RR and Rohlf FJ (1981) Biometry (2nd ed). WH Freeman, San Francisco, California.

Valerio M and Barlow GW (1986) Ontogeny of young midas cichlids: a study of feeding, filial cannibalism and agonism in relation to differences in size. Biol. Behav. 11:16-35.

Development and Physiology

DELAYED HATCHING IN THE TERRESTRIAL EGGS OF THE GRUNION, *LEURESTHES TENUIS*

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Introduction

The California grunion *Leuresthes tenuis* is among the few fish known to spawn terrestrially. Grunion (Atherinidae) are found along the Pacific coast of southern and Baja California, principally between Point Conception and Punta Abreojos (Walker, 1952). They lay their eggs on sandy beaches during spring and summer nights shortly following the new and full moons, around the time of highest high tides (Walker, 1952). The timing is important; at these times in the lunar cycle the tide is highest on the shore, so the eggs left in the sand will be out of reach of the waves until the next new or full moon. The adult grunion are deposited on the beach by surging waves and may remain out of the water for several minutes (Walker, 1952). Female grunion dig tail-first into the soft sand, and release one thousand to three thousand eggs. One or more males curl around her and release milt that flows down through the sand to fertilize the eggs (Walker, 1952). The fish then catch the next large wave to return to the sea.

The eggs are initially buried about 4 cm deep in the sand by the female, but the action of the tides can increase this depth to as much as 15 cm (Shepard and LaFond, 1940), protecting the eggs from extremes of temperature, desiccation, and predation (Middaugh et al., 1983). The eggs undergo rapid development and at 18°C are ready to hatch in about nine days, although some may hatch in as few as seven days (David, 1939). Normally, the eggs are triggered to hatch at the high tide of the next new or full moon, by waves that reach high enough on shore to carry the eggs out into the ocean (Middaugh et al., 1983; Walker 1952). If, however, the tides do not rise high enough, the eggs are able to remain viable in the sand for at least two more weeks (Walker, 1952). Thus the grunion have the ability to delay hatching. Instead of hatching at a predetermined time set by an internal developmental clock, hatching is triggered by an external, environmental factor, analogous to the situation in some terrestrially spawning frogs (Petranka et al., 1982).

The delayed hatching period requires the embryo to grow quickly to be ready for hatching at the next high tide, but at the same time, it must conserve energy to allow survival if it is forced to wait longer for a high enough wave to arrive. A similar dilemma is seen in the frog *Pseudophryne bibroni* which lays its eggs terrestrially in areas that are later temporarily flooded by winter rains (Bradford and Seymour, 1985). In this frog, eggs develop rapidly and metabolism increases up to the time that hatching is first possible, but if hatching does not occur, then the metabolism levels off and remains steady until hatching is triggered. Our hypothesis is that the grunion eggs will show a similar pattern of metabolic rate increasing during normal development and stabilizing for the delayed hatching period. We also examined newly hatched larvae of different incubation ages for body length, lipid reserve, and state of development. This is the first study to measure the metabolic rate of grunion eggs during development. Previous studies have catalogued the normal development of the grunion (David, 1939) and the influences of light (McHugh, 1954) and temperature (Hubbs, 1965; Ehrlich and Farris, 1971; Reynolds et al., 1976) on development, but these are the first data on energy requirements of the developing egg at different embryonic ages.

Materials and Methods

Individual *Leuresthes tenuis* were collected by hand (California Fish and Game permit #2826 to KLM) on the beach in Malibu, California USA in summer of 1995 as they emerged from the water to spawn. The fish were placed in a bucket of aerated seawater, and within ten minutes were stripped of eggs or milt. The eggs of each female were combined with the milt of one male in individual plastic containers holding small amounts of natural sea water. We allowed fifteen minutes for fertilization to occur, then added a small amount of sand to each container to protect the eggs during transport to the laboratory. Within two hours after collection, the eggs were placed at 18°C, a temperature appropriate for their development (Hubbs, 1965; Ehrlich and Farris, 1971). After approximately 12 hours, the eggs were rinsed free of sand and sorted.

On the day following collection, 220 eggs from each clutch were placed in a glazed porcelain combustion boat (97 x 16 x 10 mm). Small glass beads were added as a sand surrogate (0.5 mL), to hold the eggs slightly separate, wick interstitial water, and permit circulation of air. The boats were covered with cheese cloth and placed above damp sand in an opaque plastic container with holes drilled in the lid. The lid was loosely covered with a damp cloth to maintain a humid environment. Eggs were examined daily using a dissecting microscope, and any unfertilized eggs were removed at day 5. Eggs that did not show a beating heart, easily visible after day 3, were presumed dead and removed daily after day 5. Eggs were maintained at 18°C except when being examined under the dissecting microscope. Dilute sea water (20 ppt) was dropped onto the eggs by pipette when the surrounding glass beads dried out, approximately once or twice a week.

The ceramic boats (n = 10 for day 2, decreasing to n = 2 by day 23), each initially containing 220 eggs, were placed individually in 120 ml glass syringes. These respirometry syringes were then filled with room air and sealed with a three-way valve. After 20 minutes, 20 mL of air was drawn into a sample syringe. This was injected into a baseline dry air stream, passed through Drierite and Ascarite to remove water and carbon dioxide, then drawn through an S-3A Ametek Oxygen Analyzer using a flow rate of 50 ml per minute. The oxygen levels of the samples were compared to a baseline of dry room air using the Sable Systems Datacan V data acquisition and analysis program. Oxygen consumption of each egg mass was measured three times each morning throughout development, daily from days 2 (36 hours) through 23. The respirometry syringe was left open between runs for five minutes and, before closing, pumped ten times to insure equilibrium with the room air. Some mortality occurred, probably due to infection. If mortality reached 15%, in one egg mass, it was removed from the remainder of the experiment. Data were calculated per egg so that comparisons could be made across days with differing egg numbers.

Length and oil droplet size measurements were made on the larvae using a computer digitizing system, a Macintosh IIsi computer with image capture board (Quick Capture, Model DT 2255 - 50 Hz, Data Translation, Rockaway, NJ) with NIH Image 1.41 software (public domain: National Institute of Health), connected to a digital video camera (Model JE3462RG, Javelin Electronics) attached to an Olympus dissecting microscope. Larval length was measured from tip of the snout to the end of the vertebral column, excluding the caudal fin. The roughly spherical oil droplets were measured across the longest and shortest visible diameters. These two measurements were then averaged and volume was determined as if this were the diameter of a perfect sphere. Oil droplets were also measured in 20 eggs on day 4 (the first day on which the multiple oil droplets of most of the eggs had coalesced into one large drop) and in unhatched eggs.

Results

Rate of oxygen consumption ($\dot{V}O_2$) increased from day 2 through day 8 of development (figure 1). The means of daily data points for days 2 through 8 were used to establish a regression line for $\dot{V}O_2$ ($r^2 = 0.923$). At day 8, the first day on which hatching was observed, $\dot{V}O_2$ leveled off. When the means of daily data points were analyzed from days 8 through 23, the slope was not significantly different from zero (figure 1).

The volume of oil in each egg was estimated by assuming a perfect sphere, and the average volume of each group (n=14) of 24 eggs was plotted versus age of the eggs in days (figure 2). The linear regression ($r^2 = 0.808$) shows a loss of -0.004 μl per day. The x-intercept at day 22

indicates the point when the egg would be expected to run out of its lipid reserve. The lengths of the larve (n=15 groups of 24) ranged from 6.5 to 7.6 mm, but there is no significant correlation ($r^2 = 0.035$) between these size variations and the day on which hatching occurred.

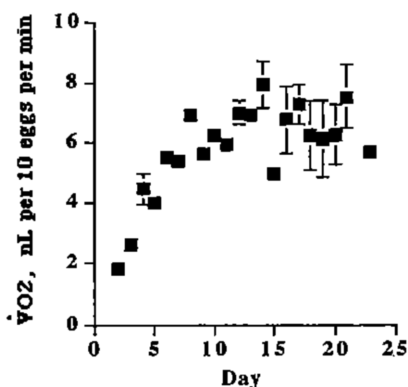

Figure 1. Oxygen consumption during development and delayed hatching.

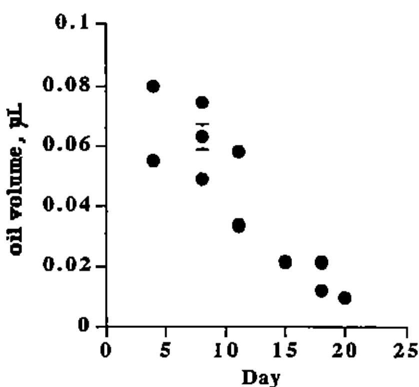

Figure 2. Oil droplet volume during development and delayed hatching.

Discussion

From the time of fertilization through day 8, the first day hatching is possible, metabolic rate of eggs increases steadily (figure 1). After that point, during the delayed hatching period, the metabolic rate stabilizes (figure 1). Since the metabolic rate remains high, the embryo does not go into a state of metabolic arrest (Hochachka and Guppy, 1987). These results are comparable to Bradford and Seymour's (1985) findings with the terrestrial eggs of the frog *Pseudophryne bibroni*, that show a steady increase in $\dot{V}O_2$ until shortly before the time that hatching first becomes possible, then level off during the delayed hatching period.

Along with the stabilizing of the metabolic rate after day 8, the length no longer increases (figure 2). Grunion embryos reach their full length at 108 hours (4.5 days) at 18°C (David, 1939). Once hatching is possible, there is no correlation between length and duration of incubation. It seems reasonable to assume that maintenance requriements alone, as one might expect if growth were no longer occurring, might take less energy than the combination of growth and maintenance activity that would occur on days 6 through 8. Nevertheless, metabolic rates level off but don't decrease in the period following day 8 (figure 1). The steady diminution of the lipid droplets of the eggs throughout development (figure 2) supports the metabolic data, and is another indication that the developing grunion expend a large amount of energy during the delayed hatching period.

Ehrlich and Farris (1971) observed no gross morphological differences between larvae that hatched after different times of incubation. However in this study, when embryos of ages that diffferred by approximately two weeks were hatched on the same day and compared to each other, internal organ development appeared different. The larvae that spent a longer time incubating before hatching seemed to be at a more developed state. Because only a small number of larvae were compared in such a fashion, these results are not conclusive, but they suggest that development as well as maintenance of existing tissue continues during the delayed hatching period. A similar pattern is seen in the eggs of the frog *P. bibroni* which compromise between energy conservation and the advantages of hatching at a more advanced stage of development by continuing to grow slowly during its delayed hatching period (Bradford and Seymour, 1985; Seymour et al., 1991). Unlike the grunion, though, *P. bibroni* continues to increase in body size. We believe the metabolism of grunion eggs through the periods of normal development and delayed hatching is similar to the metabolism during delayed hatching of *P. bibroni*, and is an example of convergent evolution across two classes of vertebrates.

It is interesting to note that, although the eggs should run out of lipid reserves around day 22, some eggs do survive and hatch after this point. Eggs in the field may be susceptible to diseases and predation by birds and mole crabs, seen in abundance at field sites. These factors, disease and predation, might warrant the prediction that, in general, the sooner an egg can hatch, the better. But if the waves do not come, the grunion have, in a sense, taken out an insurance policy and can afford to wait several more weeks for the next set of high tides to arrive.

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Literature Cited

Bradford, David F., and Roger S. Seymour. 1985. Energy conservation during the delayed-hatching period in the frog *Pseudophryne bibroni*. Physiological Zoology 58: 491-496.

David, Lore R. 1939. Embryonic and early larval stages of the grunion, *Leuresthes tenuis*, and of the sculpin, *Scorpaena guttata*. Copeia. 1939: 75-80.

Ehrlich, Karl F., and David A. Farris. 1971. Some influences of temperature on the development of the grunion, *Leuresthes tenuis*. California Fish and Game 57: 58-68.

Hochachka, P. W., and M. Guppy. 1987. *Metabolic Arrest and the Control of Biological Time.* Cambridge: Harvard University Press.

Hubbs, Clark. 1965. Developmental temperature tolerance and rates of four southern California fishes, *Fundulus parvipinnis*, *Atherinops affinis*, *Leuresthes tenuis*, and *Hypsoblennius* sp. California Fish and Game 51: 113-122.

McHugh, J. L. 1954. The influence of light on the number of vertebrae in the grunion, *Leuresthes tenuis*. Copeia 1954: 23-25.

Middaugh, D. P., H. W. Kohl, and L. E. Burnett. 1983. Concurrent measurement of intertidal environmental variables and embryo survival for the California grunion, *Leuresthes tenuis*, and Atlantic silverside, *Menidia menidia* (Pisces: Atherinidae). California Fish and Game 69: 89-96.

Petranka, J. W., J. J. Just, and E. C. Crawford. 1982. Hatching of amphibian eggs: the physiological trigger. Science 217: 257-259.

Reynolds, William W., Donald A. Thomson, and Martha E. Casterlin. 1976. Temperature and salinity tolerances of larval California grunion, *Leuresthes tenuis* (Ayres): a comparison with gulf grunion, *L. sardina* (Jenkins and Evermann). Journal of Experimental Marine Biology and Ecology 24: 73-82.

Seymour, Roger S., Fritz Geiser, and David F. Bradford. 1991. Metabolic cost of development in terrestrial frog eggs (*Pseudophryne bibronii*). Physiological Zoology 64: 688-696.

Shepard, F. P., and E. C. LaFond. 1940. Sand movements along the Scripps Institute Pier. American Journal of Science 238: 272-285.

Walker, Boyd W. 1952. A guide to the grunion. California Fish and Game 3: 409-420.

OOGENESIS IN *Sparus aurata* L.

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Abstract

The evolution of gonad activity in *Sparus aurata* L., an hermaphrodite species, is dependent on the age and growth of the fish. In most part of the one year old population sperm reabsortion is followed by oocyte maturation. Dynamics of oocyte development and related sequential cytological events were followed. The characteristics of the oocyte during the first meiotic prophase are described using electron microscopy. Ultrastructural modifications of the nucleus and cytoplasm of the oocyte were found to be linked to the different stages of the secretory activity, and with the transport and incorporation of vitellogenin by the oocyte. The existence of endocytic compartments and an highly specialised cortex allows the internalisation of vitellogenin. The study indicates that the *Sparus aurata* oogenesis like most vertebrates depends on the structural evolution of the organelle connected with the endocitic activity of the cell. At maturation and ovulation, the oocyte contains an enormous amount of reserves stocked as macromolecules, for later utilisation by the embryos.

Introduction

The hermaphrodite protandric species, *Sparus aurata* L.,a marine teleost spawning pelagic eggs, presents particular aspects of sex determination and gonad differentiation. The sex reversal process inhibits the fertility of females, and only older animals are functional females. The fecundity of females is limited by the evolution of oogonia to oocyte maturation and ovulation.The morphological changes that occur in the oocyte during the meiotic prophase and the dynamic aspects of it's growth, were used to indicate the receptivity of the oocyte to external factors that can induce final maturation and ovulation. After the reabsorption of the spermatozoa by the Sertoli and epithelial cells, during the second or third year , part of the population became functionally female (Zoar et al.1978). In fact only these phenotypic females can synthesise the yolk protein precursor internalised in the oocyte by receptor-mediated ligants (Goldstein et al.1982). This gonadotrophic-dependent phase takes place after the structural evolution of the oocyte organelle during the first meiotic prophase (Anderson,1967, Lam, 1982; Truslé et Bruslé, 1983).

Materials and methods

508 specimens of *Sparus aurata* were collected in the Algarve, south of Portugal, and in the Obidos lagons using trammel nets in different months of the year. Age determinations were made by direct readings of the scales. The fork length (o,5cm) and weight (g) were correlated with age. Specimens with different ages collected from the wild using trammel nets, and specimens maintained in laboratoy conditions were killed and selected organs were used for histological study. After macroscopic

observation, the gonads were sectioned into pieces measuring 0,5 cm in diameter these tissues were prepared for light microscopy, to determine the distribution of male and female germinal tissues. Small pieces of the same gonads were (fixed in 3% glutaraldehyde, sodium cacodylate 0,1 M and 0,05 $CaCl^2$, for 3 hours, rinsed in buffer and post-fixed in 1% osmium tetroxide 1h for dehydratation) for electron microscopy studies. The tissue was embedded in Epon. The thin sections were stained whit uranyl acetate followed by lead citrate and were examined with a transmission electron microscope.

Results

Physiological state of the oocytes

The nucleolus

In the germinal epithelium of *Sparus*, oogonia with a compact nucleolus originate mitotically oocytes at first meiotic prophase. A basement membrane and rare follicle cells involve the leptoten-pachyten oocyte. First the nucleus presents a chromosome pairing appearance, the synaptonemal complex. Later the nucleolus establishes specific relations with the nucleolar organiser region (NOR) (fig.1) .At diplotene the nucleolus enlarges, presenting a central fibrilar core and a granular periphery.Multiple nucleoli, in number of twenty, disconnected from the nuclear envelope are nucleolus with an outer granular layer and granules dispersed in the nucleoplasma, or associated with nuclear pores are observed. At late dictyate (Fig.2) the oocyte contains a nucleolus that has ceased growing and has decreased in size. Some spherical nucleoli containing vacuoles remain at the periphery.

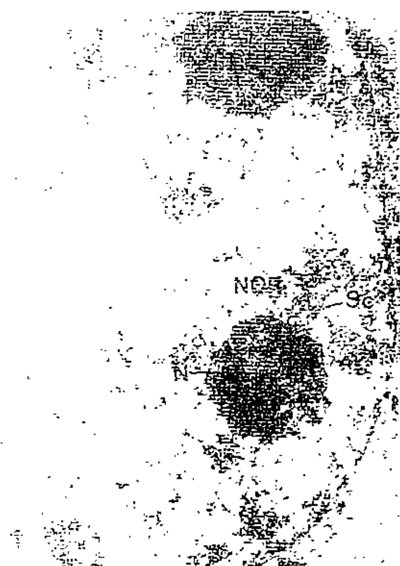

Fig.1 Leptoten-Pachitene-oocyte.Nucleolus (N) associated to a synaptonemal complex (Sc),nuclear organiser region (NOR) 12000x.

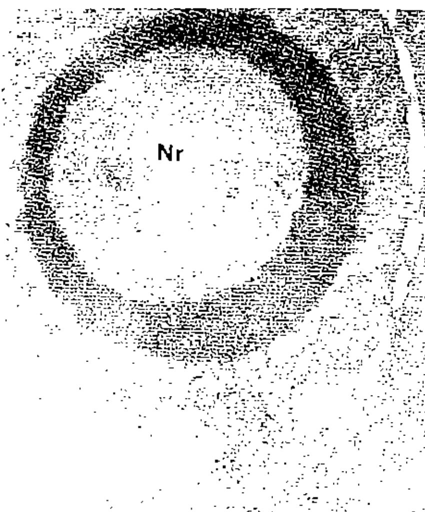

Fig.2 Diploten nucleus shows a ring shape (Nr) 36000x.

Nucleolus-cytoplasmic interaction

The RNA processing takes place during the migration of the molecules from the site of synthesis, crosssing the nuclear envelope and accumulated in the large number of ribosomes which appear in the cytoplasm before endocytic accumulation of yolk. Granulo fibrilar mass, nucleolus-like bodies (NLB) were observed in all stages of oogenesis in *Sparus* (Fig.3).

The cytoplasm. Endocytic activity

The endocytic activity in three to four year old females takes place in the oocyte, after RNA accumulation. Endoplasmic reticulum vesicles and elongated mitochondria are dispersed in the cytoplasm, occasionally associated with microfilaments. Golgi stacks are predominantly in a peripheric position near multivesicular bodies (MVB). Lisosoms appear in the cytoplasm. Lipid and cortical alveoli are elaborated. Microvilli originated by protusion of the oocyte oolema in the interfollicular space and the microvilli of cells completely surrounds the oocyte (fig.4).

Fig.3 Nucleus(N) granulo fibrilar mass, the nucleolus-like bodies (NLB) nuclear pore (Np) 36000x.

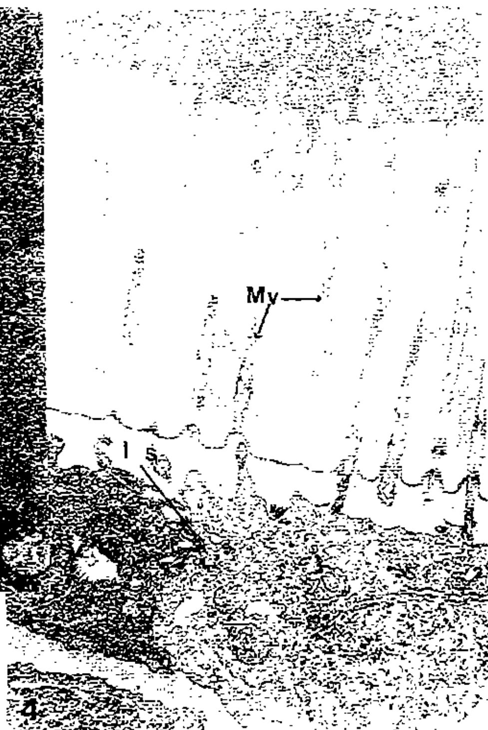

Fig.4 (Mv) Microvoilosities. interfollicular space (Is) 30000x

The different layers of polyssacarids deposited between the pré existent microvilosities form the zone pellucid. Microfilaments of 6nm are observed in the cytoplasm. Pinocytosis is initiated at clathrin coated regions of microvilli which pinch off to form coated vesicles (Fig. 5) These vesicles lose their coats and deliver their contents at endosomes. These transfer the content to the lisosomal compartment, and after hydrolysis form the yolk spheres (Fig.6).

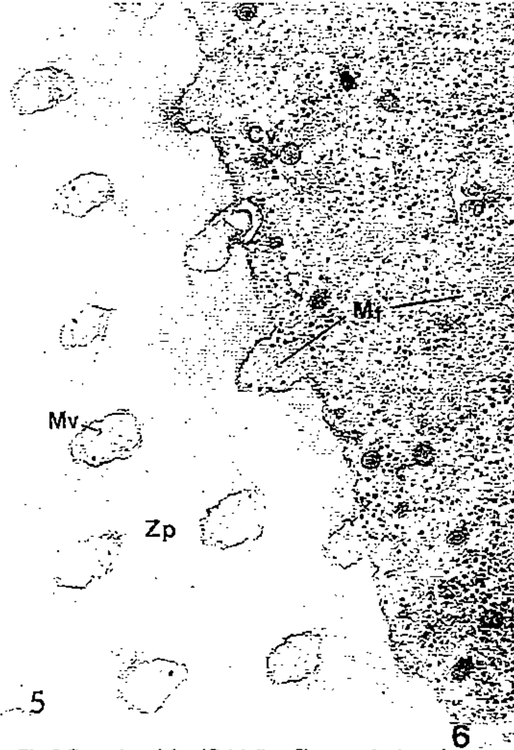

Fig.5 Coated vesicles (Cv).Microfilaments in the cytoplasm (Mf),microvilosities(Mv). Zone pellucide (Zp).40000x.

Fig.6Yolk spheres are observed (Y) 16000x

After using HCG stimuli at 18-20°C the vitellogenesis is completed in a few hours. The full grown oocyte shows the germinal vesicle excentraly located. The nuclear envelope disrupts after forming several infoldings. Following metaphase II the microvilli are in reabsorption the (7) follicle cells degenerate and disperse from the zone pellucida. The yolk components are agglutinated and in a continuous cortex layer. At the oocyte periphery cytoplasmic organelles are lacking, and only the cortical alveoli are found (8). After hydration, ovulation occurs, and eggs float in sea water prepared for fertilisation.

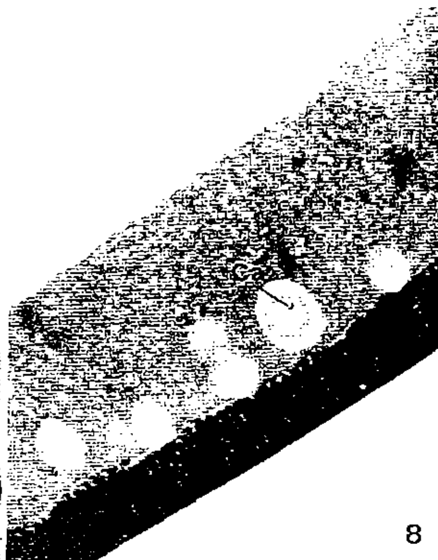

Fig.7 Microvilli (Mv) are reabsorved. Follicle cells degenerate (Fc) 40000x.

Fig.8 Cortical alveoli (Ca) are at the periphery. 16000x.

Discussion

After leptotene-pachitene in the oocyte evolution, occurs specific relations of the nucleolus with the the NOR that contains the rDNA with transcritional units for the synthesis of 18s and 28s ribosomal RNA (Goessens,1984).The RNA synthesis and accumulation is possible due to the amplification of the genes responsible for the rRNA organisation, and it takes place during oocyte growth. The protein synthesis for posterior embryonic differentiation, depends on the existence of a large number of ribosomes and RNA transfer (Denis,1977). The RNA 5s produced during the first growth and are 75-80% of the ribosomic content of the primary oocyte. During vitellogenesis the oocyte produces mainly RNA 28s (Denis,1977).The nuclear envelope has the function of compartmentation in space during the different stages of the oogenesis, and separates transcription of protein synthesis. Associated with spherical mitochondria, in leptoten-pachytene the NLB has been identified cytoplasmicaly in other species of fish as ribonucleoprotein with nuclear origin (Toury, et al. 1977; Azevedo, 1984). At diplotene the NLB is perinuclear, but mitochondria are dispersed in the cytoplasm. The endocytic activity of *Sparus aurata* oocyte, depends directly on the structural evolution of the organelle connected with metabolic activity, and on the differentiation of the zone pellucida, follicle and theca cells. It depends indirectly on the synthesis of phospholipoproteins by the liver, and its transport and incorporation into the oocyte.A functional three to four year old female with an asynchronous ovary, presents oocyte maturation according to a circadian rhythm. The diplotene dictiate in *Sparus* oocytes has a long duration (Ramos,1986).The one to two year old fish do not enter into vitellogenesis, at least the accellular layers of zone pellucida are not deposited, and probably the folllicule cells do not yet synthesise the oestrogen necessary for stimulation of the liver to produce the yolk protein precursor (Aida et al. 1973). The oocyte morpholoy of older fish shows an highly specialised cortex and the existence of endocytic compartments which at this stage, allows that the vitellogenin to be taken in large amounts (Routh et Porter 1962;Goldstein et al,.1982; Mabilot,1984; Selman et Wallace 1982) passing through the intercellular space of the follicular epithelium (Abraham et al. 1981). The present study indicates that the *Sparus aurata* oogenesis, like the most in other oviparous vertebrates depends on the structural

evolution of the organelle connected with the auxocitosis and with the endocitosis of the cell. At maturation and ovulation, the oocyte contains an enormous amount of reserves socked as macromolecules, for later utilisation by the embryos.

References

Abraham, M., Hilge, V., Lison, S.,Tibika, H.& Rahamin, E., 1981 The envelope cells of oocytes and pathway of intravenously injected HRP in the teleostean ovary. The European Society for Comparative Endocrinology. XI Conference, (proceedings) Jerusalém. 120-121.

Aida, K., Hirose, K., Yokote, M. & Hibuiya, T, 1973 Physiological studies on maturation of fishes -II Histological changes in the liver cells of Ayu following gonadal maturation and estrogen administration. Bull.Jap. Society of Scientific Fisheries. 39 (11),1107-1115.

Anderson, E., 1967 The formation of the primary envelope during oocyte differentiation in Teleosts. J. Cell Biology. 35,193-212.

Azevedo, C., 1984 Development and ultrastructural autoradigraphic studies of nucleolus like bodies (nuages) in oocyte of viviparous teleost (*Xiphophorous helleri*).Cell Tissue Res. 238;121-128.

Bruslé,J.; Bruslé, S., 1983 Gonadogenesis in fish. Can.transl.Sci.Fish Aquat.Sci.5025,58.

Denis, H. , 1977 Accumulation du RNA dans les oocytes des vertebrés inferieurs. Biol. Cellulaire.28,87-92.

Goessens,G.,1984 Nucleolar structure. Int. Rev. Cyt. 87,107-158.

Goldstein, J.L., Anderson, R.G.W. & Brown, M.S.,1982 Coated vesicles and receptor mediated endocytosis. Nature.279,679-685.

Lam, T.,1982 Applications of endocrinology to fish culture . J.Fish Aquat. Sci. 39,111-137.

Mabillot,S.B.,1984 Endosomes transfer yolk proteins to lisosomes in vitellogenic oocyte of trout Biol. Cellulaire. 51,53-66.

Ramos, M.A. ,1986 Contribuição para o conhecimento da ultrastrutura do oocito e do funcionamento da gónada de *Sparus aurata* L. (Pisces Perciformes). Thesis, INIP. 123.

Roth, T.F., Porter, K.R.,1962 Yolk protein up-take in the oocyte of mosquito *Aedes aegypti*. J.Cell Biol.20, 313-331.

Selman, K.; Wallace, R.A., 1982 Oocyte growth in the sheepshead minnow:Uptake of exogenous proteins by vitellogenic oocytes. Tissue and Cell. 14,3,555-571.

Toury R., Clérot, J.C., André, J. 1977 Les groupesments mitochondriaux des cellules germinalesm des poissons téleosteens Cyprinidés du ciment intermitochondrial isolé. Biol. Cellulaire. 30, 225-232.

Zoar Y., Abraham, M., Gordin, H.,1978 The gonadal cycle of the captivity reared hermaphroditic teleost *Sparus aurata* L. during the first years of life. Ann.Biol. anim. Bioch. Biophys.18 (4), 877-882.

DEVELOPMENT AND ENERGY UTILIZATION IN EARLY LIFE STAGES OF VIVIPAROUS YELLOWTAIL ROCKFISH

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ABSTRACT

Laboratory experiments were conducted with gestating adult yellowtail rockfish and field surveys done of females in late vitellogenesis and gestation to determine the rate of embryonic and larval development, to examine the different measures of egg quality, and to determine the rate of endogenous energy utilization and tissue assimilation. Embryogenesis and larval development followed a predictable sequence and showed rapid development with hatching occurring in 23d and parturition in 29d. Eggs varied in dry weight and energy content with lipid concentrations most directly contributing to the amount of energy per egg. The endogenous energy of yolk and oil globule declined rapidly throughout gestation resulting in limited yolk and oil reserves at parturition. Findings of this research will serve to allow estimation of stock biomass and will contribute to the understanding of the underlying factors that contribute to recruitment variation.

INTRODUCTION

A unique trait common to all *Sebastes* species is their live-bearing reproductive strategy which can be classified as ovoviviparous or viviparous (Wourms, 1991). It is important to know the time course and sequence of embryonic and larval development to enable fishery researchers and managers to estimate adult rockfish biomass by means of the larval production method (Lo et al., 1992). Further, early life stage physiological condition and nutritional and energetic states may play important roles in determining the success or failure of yearclasses, an often cited argument presented to explain recruitment variability (Houde, 1987). A study of a ten year trawl and diving survey of pelagic and newly settled juvenile yellowtail rockfish concluded that population regulation is based on dynamics that influence larval growth and survival (Ralston and Howard, 1995). To address the applied needs for population assessment and to better understand factors effecting early life stage growth and survival a combined field and laboratory study was conducted on yellowtail rockfish (*S. flavidus*) from waters off northern California.

The yellowtail rockfish is a semipelagic, viviparous species that is commercially important through most of its range along the Pacific coast of the United States. Reproductive biology of this species has been the focus of research for approximately the last ten years. The annual reproductive cycle and variability in reproductive effort of the yellowtail rockfish have recently been established (Eldridge et al., 1991, Eldridge and Jarvis, 1995). Maternal nutritional dynamics and their relationship to reproduction and gonadal condition were described and analyzed by Norton and

MacFarlane (1994). Reproduction of this species, and most likely other rockfishes, has been found to be spatially and temporally variable, in both quantitative and qualitative measures, and it is closely related to environmental conditions and to reproductive success.

Previous ontogenetic studies of early life stages of *Sebastes* species are limited. Only Yamada and Kusakari (1991) provide descriptions from controlled experimental conditions using the *S. schlegeli* of Japan as a model. Information of different aspects of embryonic and larval rockfish energetics was reported in studies by Boehlert et al.(1986) and Boehlert and Yoklavich (1984).

The research design of this study combines results from field surveys of adult yellowtail rockfish collected by hook-and-line off northern California with those from laboratory experiments of spawning adults held in captivity. The objectives of this research were to a) describe the time-course and sequence of embryonic and larval development during gestation in the yellowtail rockfish, and b) to examine measures of egg quality, in primarily nutritional and energetic terms, and to determine how endogenous energy stores are utilized during early life stage development.

MATERIALS AND METHODS

For the early life stage study, 21 fertilized female yellowtail rockfish were collected from 1990 - 1993 by hook-and-line from Cordell Bank, approximately 37 km off the California coast, and transported live to the University of California Bodega Marine Laboratory. Fish were maintained in 2000 L circular tanks at ambient light and water conditions for the duration of the study. Temperatures averaged 11.96 degrees Centigrade (SD = 0.88°C) and salinities averaged 32.31 ppt (SD = 0.78 ppt). The average size of the gestating females was 37.8 cm SL (SD = 3.8 cm). At three day intervals throughout the study, which lasted from December through March of each year, fish were anesthetized and a catheter inserted through the urogenital papilla and into the ovary to obtain samples of developing oocytes. Each sample was examined fresh by microscope after removal from the host, and the developing embryo or larva assigned a developmental stage according to the developmental series established by Yamada and Kusakari (1991). This scheme ranges from 1 (unfertilized oocyte) to 33 (larva at parturition).

A corresponding field study was also conducted with adult yellowtail rockfish from 1986 to 1991. From a total collection of 715 adult females taken from Cordell Bank for reproduction studies, 108 fish were found to have fertilized eggs. This latter group was selected for detailed examination of gonadal tissue and the developing embryos and larvae. The adults ranged in standard length from 29 to 46 cm and total weight from 673 to 2083 g. The ovary of each fish was assigned a macrospcopic numerical gonadal stage. The values of this five stage scheme is as follows: 1 = immature, 2 = vitellogenic eggs, 3 = fertilized eggs, 4 = spent or ovaries with recent parturition, and 5 = resting and/or recovering). In this paper, eggs from stages 2 and 3 will be used. Within these stages fractional division from 0.1 to 0.9 was assigned which represented the progressive stage of development within either vitellogenesis or embryogenesis. From each female, samples of the eggs were staged and preserved in 10% buffered formaldehyde, or frozen at -70°C. Eggs were later removed for dry weight determinations (triplicate samples of 20 eggs each, weighed to the nearest 0.0001g). Caloric content was determined by bomb calorimetry in a adiabatic bomb calorimeter. Total lipids were quantified by automated thin layer chromatography/flame ionization detection after chloroform-methanol biphasic extraction. Total protein content was determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin as a standard.

Three gestating females from the experimental group were selected for a study of early life stage energy utilization because they provided samples of eggs ranging from late vitellogenesis, prior to fertilization, through embryogenesis, hatching, and larval release. Formaldehyde fixed specimens at each sampled stage of development were dissected into chorion, yolk, and tissue portions, dried

at 100°C for 24 hours, and weighed in triplicates of 20 specimens to the near 0.0001 g. Estimates of the oil globule weight were obtained by subtraction of the combined yolk, tissue, and chorion weights from the total egg weight.

Data from this study were organized into EXCEL spreadsheets, and analyzed for curve-fits and statistical significance by Tablecurve and SYSTAT software.

RESULTS AND DISCUSSION

Embryonic and Larval Development - Of the 21 adult fish successfully held and sampled in the laboratory, 8 provided specimens that spanned from late vitellogenic, unfertilized ova to parturition. The thirteen remaining fish produced specimens that ranged in beginning stages from morula stage to embryos with optic cups and otoliths and extending on to parturition. The developmental sequence followed those of Yamada and Kusakari (1991) and Sanchez and Acha (1988), but the rate of development and the times to hatch and parturition were much more rapid (Table 1). The important developmental periods for field applications in stock assessment can be derived from Figure 1 which has a best predictive equation of $Y = (28.6133 - 0.255935X^2)$, $r^2 = .94$. The time from fertilization to larval release averaged 29.2 d, with a 5d range, most likely due to temperature variation. In contrast, the equivalent time for a much larger *S. schlegali* larva of Japan was 48 d.

Developmental Stage and Gonadal Traits - Field surveys of adult yellowtail rockfish during gestation showed that ovary size did not significantly increase in relative proportion throughout gestation ($r^2 = .09$, N.S.; Figure 2). We conclude that embryos did not demonstrate significant weight increase during gestation despite the fact that MacFarlane and Bowers (1995) did find limited matrotrophic contribution to embryonic and larval nutrition. Gonosomatic indices showed wide variation during gestation, which is probably attributable to the different ages and sizes of the host females.

Egg Quality - Qualitative measures of egg quality varied greatly among field caught females with late vitellogenic and/or early embryogenic eggs (Table 2). Eggs varied significantly in size ($P < 0.01$) and in calories/egg. Since no differences were found in the caloric content per unit weight among the eggs tested, the observed differences in the energy contents of the eggs was due to weight differences. Variation in egg size within species is common among a variety of fish species and has been found to relate to both inherent and environmental factors (Wootton, 1979). Lipids comprise the most energy rich nutrient source for development and Figure 3 demonstrates how energy content of the eggs was directly and positively correlated with the measured lipid content in the ovary ($r^2 = .46$). When the energy content of the egg was analyzed over the course of vitellogenesis and gestation, we found an increase in energy during the period of yolk deposition, prior to fertilization, and a corresponding decrease from fertilization to larval release. This pattern is in agreement with findings of Norton and MacFarlane (1994) for the vitellogenic period and with MarFarlane and Norton (1996) during gestation, both studies using analytical chemical methods.

Table 1. Sequence, time course, and corresponding stages of embryonic and larval development of yellowtail rockfish.

Developmental Stage	Yamada/Kusakari Stage	Days Post - Fertilization
Newly fertilized oocyte	1	0
Germ disc formation	2	↓
Early cell cleavage	3-8	↓
Morula	9	3
Blastula	10-11	↓
Epiboly	12	↓
Gastrula	13-14	5
Embryonic shield	15	↓
Headfold formation	16	7
Optic vesicle	17	↓
Somite formation	18	↓
Optic cups/auditory vesicle	19-22	11
Otolith formation	23	↓
Retina pigmentation	24-25	18
Blood circulation/mouth and anus open	26-29	21
Yolk depletion	30	↓
Hatching	31-32	23
Yolk and oil globule depletion	32	↓
Parturition	33	29

Developmental Period	Mean (days)	SD	Range
Fertilization to hatching	23.0	1.3	21-25
Hatching to parturition	6.2	1.8	4-10
Fertilization to parturition	29.2	2.4	27-33

Early Life Stage Conversion Efficiency - Gravimetric analyses of endogenous energy utilization (i.e. yolk and oil globule) in embryos and larvae during gestation showed steep linear declines in yolk reserves and gradual declines in oil globule reserves from fertilization to parturition (Figure 5). At the same time, an exponential increase in tissue assimilation was found with the most rapid increase occurring after approximately 14 days post-fertilization. At hatching (i.e. day 23) 40% of the yolk reserves and 26% of the oil globule remained. Overall conversion efficiency by weight from fertilization to parturition was 54%. This compares favorably with the chemical analytical approach used by MacFarlane and Norton (1996) in yellowtail rockfish and with reviews of other fish species during their endogenous feeding periods (40-70%; Blaxter, 1969).

By parturition time when larvae have been incubated for approximately 6 days, only 14% of the original yolk mass remains and 8% of the oil globule remains. These limited reserves indicate that newly released larvae must quickly convert to exogenous food resources for survival. This further supports the Ralston and Howard (1995) finding that dynamic factors influencing the early larval stage, soon after parturition, comprise the most critical time for determining year-class size.

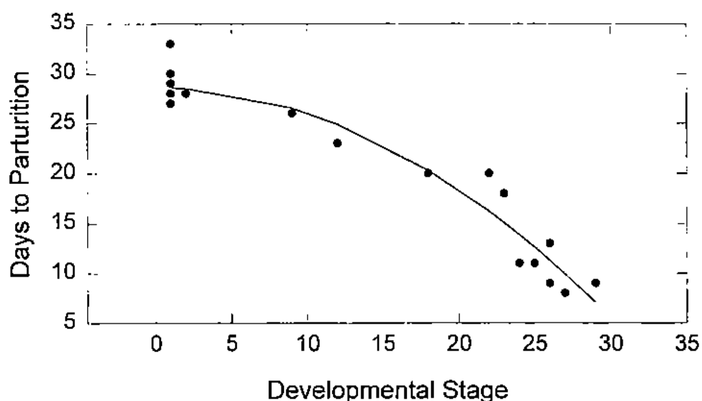

Figure 1. Developmental days to parturition.

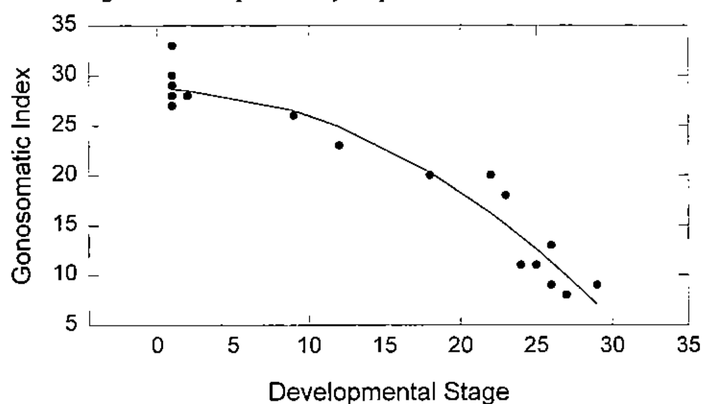

Figure 2. Gonosomatic index by developmental stage.

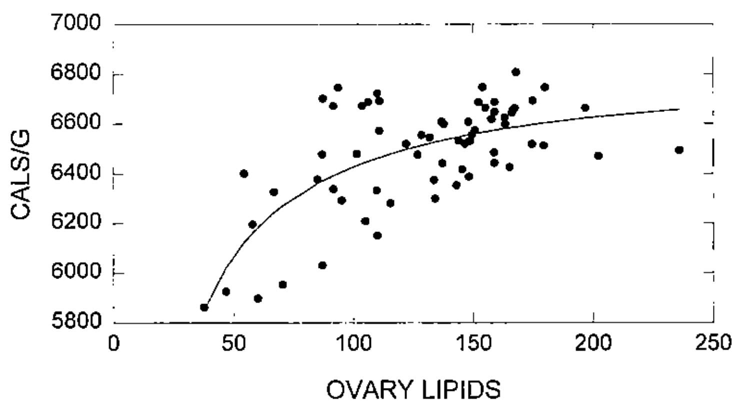

Figure 3. Caloric content and lipid concentrations.

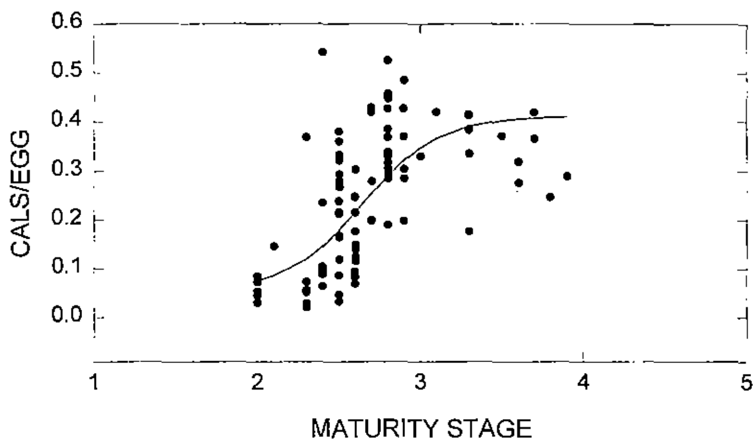

Figure 4. Energy content per egg by gonadal maturity stage.

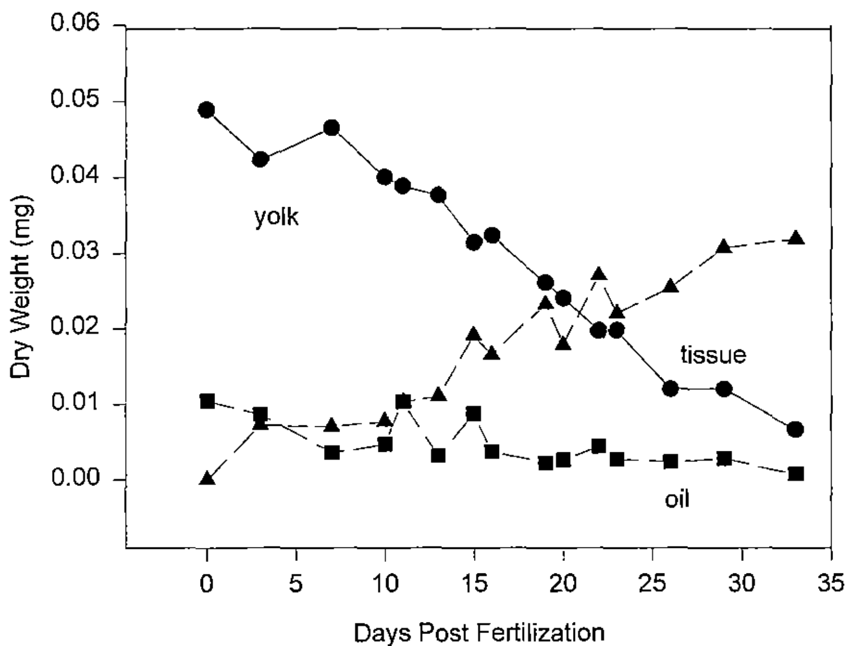

Figure 5. Dry weight of yolk, tissue, and oil globule by the days after fertilization.

Table 2. Measurements of egg quality in yellowtail rockfish.

Variable	Mean	SD	Range
Egg dry weight (mg)	.03766	.02132	.0012-.08500
Calories/g	6,465	259	5,607-6,890
Calories/egg	0.252	0.134	0.020-0.541
Ovary lipid (mg/g)	128.2	41.0	37.7-235.9
Ovary protein (mg/g)	210.2	60.5	73.8-333.0

The findings of this study provide a basis for estimating the time to larval release, essential for estimating stock biomass, and applicable to other *Sebastes* species. Variation in egg size and quality and the pattern of endogenous energy utilization leading up to the time of larval release all factor into the determination of successful recruitment.

REFERENCES

Blaxter, J H S 1969 Development: eggs and larvae. *In* W S Hoar and D J Randall (editors), Fish Physiology, Vol. III, p.177-252. Academic Press, N. Y.

Boehlert, G W, Kusakari, M, Shimizu, M, Yamada, J 1986 Energetics during embryonic development in kurosoi, *Sebastes schlegeli* Hilgendorf. J. Exp. Mar. Biol. Ecol. 101: 239-256.

Boehlert, G W, and Yoklavich, M M 1984 Reproduction, embryonic energetics, and the maternal-fetal relationship in the viviparous genus *Sebastes* (Pisces: Seorpaenidae). Biol. Bull. 167: 354-370.

Eldridge, M B, Whipple, J A, Bowers, M J, Jarvis, B M, and Gold, J 1991 Reproductive performance of yellowtail rockfish, *Sebastes flavidus*. Env. Biol. of Fishes 30:91-102.

Eldridge, M B, Jarvis, B M 1995 Temporal and spatial variation in fecundity of yellowtail rockfish. Trans. Amer. Fish. Soc., Vol. 124 (1):16-25.

Houde, E D 1987 Fish early life dynamics and recruitment variability. Am. Fish. Soc. Symposium 2:17-29.

Lo, N C-H, Hunter, J R, Moser, H G, Smith, P E, and Methot, R O 1992 The daily fecundity reduction method: a new procedure for estimating adult fish biomass. ICES Jour. of Mar. Sci. 49: 209-215.

Lowry, O H , Rosebrough, H J, Farr, A L, and Randall, R J 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

MacFarlane, R B and Bowers, M J 1995 Matrotrophic viviparity in the yellowtail rockfish *Sebastes flavidus*. Jour. of Exp. Biol. 198, 1197-1206.

MacFarlane, R B and Norton, E C 1996 Lipid and protein changes during embryo development in the viviparous genus Sebastes: application to the assessment of reproductive success. High performance fish symposium proceedings. Sponsored by the Amer. Fish. Soc. Physiology Section, July 14-18, San Francisco, CA.

Norton, E C and MacFarlane, R B 1994 Nutritional dynamics of reproduction in viviparous yellowtail rockfish, *Sebastes flavidus*. U. S. Fishery Bull. 93: 299-307.

Ralston, S and Howard, D F 1995 On the development of year-class strength and cohort variability in two northern California rockfishes. U. S. Fishery Bull. 93: 710-720.

Sanchez, R P and Acha, E M 1988 Development and occurrence of embryos, larvae and juveniles of *Sebastes oculatus* with reference of two Southwest Atlantic Scorpaenids: *Heliocolenus dactylopterus lahillei* and *Pontinus rathbuni*. Meeresforsch 32: 107-133.

Wooton, R J 1979 Energy costs of egg production and environmental determinants of fecundity in Teleost fishes. *In* P J Miller (ed) Fish Phenology: anabolic adaptiveness in teleosts. Academic Press, London, p. 133-159.

Wourms, J P 1991 Reproduction and development of *Sebastes* in the context of the evolution of piscine viviparity. Env. Biol. of Fishes 30: 111-126.

Yamada, J and Kusakari, M 1991 Staging and the time course of embryonic development in kurosoi, *Sebastes schlegeli*. Env. Biol. of Fishes 30: 103-110.

EMBRYOLOGICAL CHARACTERISTICS OF TWO *OREOCHROMIS SPP.* (TILAPIA) AND THEIR HYBRID USING THE INDUCED SPAWNING METHOD.

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Abstract

The principal diagnostic features of the embryonic and larval stages were obtained by induction of spawning of two common species of great importance in Egypt i.e. ***Oreochromis niloticus*** **&** ***Oreochromis aureus***. The well developed embryonic vascular system has attracted the attention to its relevance to the respiratory function.

The resulting hybrid has in its developmental stages, some characteristics that are half way between those of the two pure parent strains and some very similar at a corresponding stage of development.

Introduction

The genus ***Tilapia*** to which ***Oreochromis species*** is belonging to is characterized by its tolerance to different environmental conditions beside their hardness, ease of breeding, diversified habitats and fast growth.

Since this species has become popular in aquaculture around the world, more detailed information is needed about its embryological stages and development. Their sensitivity and adaptability to adverse conditions of low oxygen concentration - caused by pollutants with organic matter - should be studied.

The hybrid in Egyptian lakes dominates the total catches of fish, an investigation is needed to know to which extent it deviates away from the pure parental species.

Materials & methods

The fish were transferred prior to spawning to fiber glass tanks containing 0.4m^3 water for each group of ± 12 individuals of sizes ranging between 12-19 cm. at a water temperature between 22-

27° C. The ratio between males & females was 1:3. The experiments were done for each pure species independently as well as for the hybrid.

Induced spawning

Both Sexes were induced for spawning by injection using human chorionic gonadatrophin (H.C.G) intramuscularly.

Artificial insemination method was also applied using H.C.G hormonal injection or without it. This has been done by mixing the mature and ripe ova evacuated by stripping the fish belly towards the genital vent and adding the milt of the male directly and allow it to fertilize.

Incubation and handling of eggs and larvae:

The fertilized eggs were either left to the mother brooder to incubate it naturally (orally) or artificially using a suitable device to make the conditions as similar as the natural environment as possible.
Some eggs (3-5) are taken at intervals from the time of fertilization till hatching and then till complete absorption of yolk to study the successive stages of embryonic and larval development.

Results & discussion

The aim of using H.C.G. is to accelerate the oocyte maturation and the ovulation in both species. The response to the hormone treatment depends on the degree of maturity; some fish responded after ± 10 hours post injection while part of the rest responded after the second injection and the others did not respond at all.

Attention has been paid to the principal diagnostic features of the embryonic and larval stages. This includes division of blastomeres and formation of blastoderm, the expansion of blastoderm over the yolk sphere (epiboly), development of central nervous system, pigmentation, formation of somites and their number, optic and otic development, heart development and blood circulation (plates I & II). Complete information on those measures and values at the time of hatching and at 24 hours after hatching are shown in the table 1.

Measures at the time of hatching			
Measurement	*O. aureus*	*O. niloticus*	Hybrid
Age at hatching	86 hours	80 hours	76 hours
Temperature	27°C- 29°C	28°C	29°C
Completion of hatching	97 hours	96 hours	88h +/- 2h.
Total length	4.6-4.8 mm	4.56 mm	4.7-5.0 mm
No. of segments in the trunk region	19	17-19	18
No. of segments in the tail region	14	12-13	13
Height of the head	0.8 mm	0.62 mm	0.49 mm
Inter orbital	0.4×0.4 mm	0.62 mm	0.49 mm
Distance between eye margin and auditory capsule	0.25	0.15	0.38
Egg yolk diameter	2.1× 2.3	2.4× 2.4	2.6× 2.8
Measures at 24 hours from hatching			
Body length	5.8 mm	5.9 mm	5.45 mm
No. of segments in the trunk region	15	17-19	19
No. of segments in the tail region	14	12-13	14
Height of the head	1.1 mm		085 mm
Inter orbital	045 mm	0.5 mm	0.45×0.4
Distance between eye margin and auditory capsule	0.2 mm	0.26 mm	0.15 mm
Egg yolk diameter	1.7× 1.9	2.5	2.35×2.65

Table 1. Measurements taken for the pure species and the hybrid at two different stages.

Embryonic blood vessels and their respiratory function

The erythrocytes appears and develops very early in the embryonic stages i.e. about 38 hours before hatching stage (plate II) and continues till hatching stage (plate III).

The embryonic respiratory blood vessels reaches its peak of development during the first day after hatching (age 104 hours ± 4 hours) for both pure species. In the head region blood passes in the mandibular vessel while in the trunk and tail it passes in the dorsal aorta, caudal artery and caudal vein. The subintestinal vein is branching to collect in the subintestinal vitelline vein that surrounds the yolk sac with a network of blood vessels that cover the entire surface of the yolk sac (plates III & IV). In this respect we may notify that the developed embryonic vascular system is thought to be a way of adaptation that suits the condition of low oxygen concentration. The species under investigation can survive in habitats of poorly oxygenated areas; hence the nature and development of the embryonic respiratory vessels in ***Oreochromis spp.*** are correlated with low oxygen concentration.

Some Results on the Hybrid:

The offspring resulting from crossing ***O. aureus*** (female) and ***O. niloticus*** (male) have in its embryonic and larval stages some characteristics half way between those of the two parents and some are very similar to those of the parent species. One of the most frequently used characteristics is the duration of the embryonic developmental period starting from the time of fertilization till the hatching stage. The resulting hybrid hatch between 76 - 88 hours from fertilization, while for ***O. aureus*** and ***O. niloticus*** was 86 - 97 hours and 80 - 96 hours respectively under the same laboratory conditions of temperature and incubation.

The length of the resulting hybrid embryo; at the time of hatching was 4.7 - 5.0 mm. when the yolk sac dimensions were 2.6 × 2.8 mm. For the pure breeding of ***O. aureus*** the length was 4.6 - 4.8 mm. when the yolk sac was 2.1 × 2.3 mm., and 4.5-4.7 mm. for ***O. niloticus*** when its yolk sac was 2.4 × 2.4 mm in diameter. This indicates that the length of the embryo during development depends mainly on the yolk sac dimensions. In this respect, the length of the embryo is relative to the yolk sac dimensions and show that some parental factors (the female gamete) was influencing its rate of growth. This agrees with Peter's observation (1963) that the overall size of the larva, and hence the head length, depends on the original size of the fertilized egg and its quantity of yolk. This in turn depends upon the size of the mother fish. This fact was also emphasized by Nussbaum & Chervinsky (1968) on their study on artificial incubation of ***Tilapia nilotica***. This observation shows that the intermediary is not a basic characteristic for the hybrid but is biased to the maternal gamete supported by the investigations of Ross and Cavender (1981); Barret (1983); Leary et al., (1985). These authors had pointed out that not all characters necessarily show such pattern of morphometric and meristic intermediary. They added that the intermediary hypothesis presupposes a genetic mechanism involving blending inheritance; given the probable operation of other genetic systems (e.g., dominance, epistasis; etc.). Occurrences of non- intermediary in at least some characters should not be surprising (Jeffrey et al, 1986).

More studies are needed to know the rate of growth in the post larval stages and fingerlings of the hybrid in comparable to the pure breed species and to clarify to what extent is the hybrid can tolerate the adverse conditions and its adaptability with different conditions.

References

Barret, P.J (1983) : Systematics of genus Tilapia (perciforms: Cichlidae) in the lower Colorado River basin. Msc. thesis, Arizona state Univ., Tempe, Arizona.

Jeffrey N. Taylor, David B. Snyder and Walter R. Countenay, Jr. (1986) : Hybridization between two introduced, substrate - spawning Tilapias (Pices: Cichlidae) in Florida. Copeia pp. 903-909..

Leary, R.F., F.W. Allendorf and K.L kundsen. (1985) : Developmental instability and high metric counts in interspecific hybrids of Salmonid fishes. Evolution 39 (6):1318-1326.

Nussbaum, M., and Chervinsky J. (1968) : Artificial incubation of ***Tilapia nilotica***. Bamidgh 20 (4) : 120-124.

Peter, H.M., (1963) : Eizahl, Eigewicht und Geleg. Entwicklung in der Gattung Tilapia (Cichlidae, Teleostei) Int. Revue Ges. Hydrobiol., 4:233-236.

Ross, M.R., and T.M. Cavender. (1981) : Morphological analysis of four experimental intergeneric Cyprinid crosses. Copeia 1981: 377-387.

List of abbreviations in the plates

Au C	Auditory Capsule
Au P	Auditory pit
B	Brain
BA	Branchial Artery
CA	Caudal Artery
CD	Cuvierian Duct
CV	Caudal Vein
DA	Dorsal Aorta
E	Eye
EM	Egg Membrane
H	Heart
I	Instestine
M	Myotomes
P	Pigments
SIV	Sub-intestinal Vein
VV	Vitelline Vein

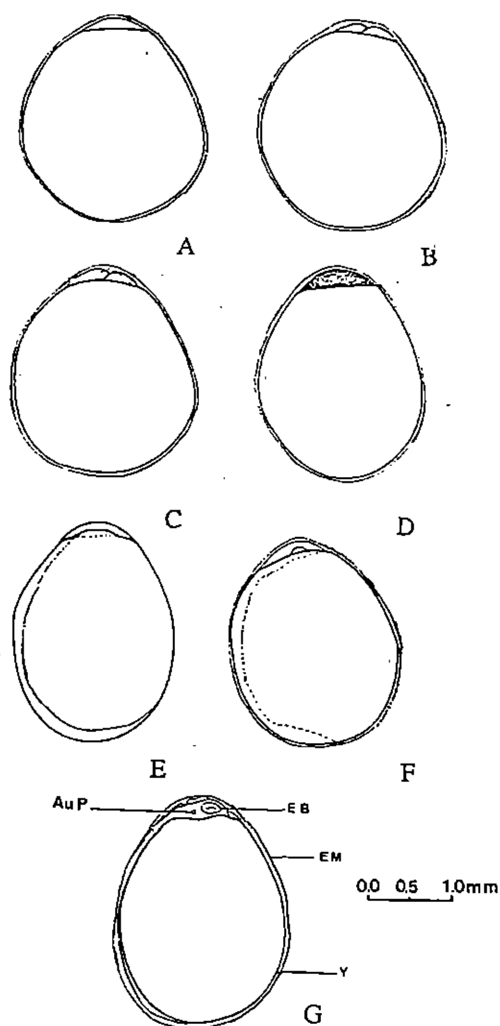


Plate I. Early stages of *Orechromis niloticus*

A- Germinal disc stage
B- Two-cell stage
C- Four-blastomere stage
D- Morula stage
E- End of gastrulation
F- Begining of Organogenesis
G- 27 hours from fertilization

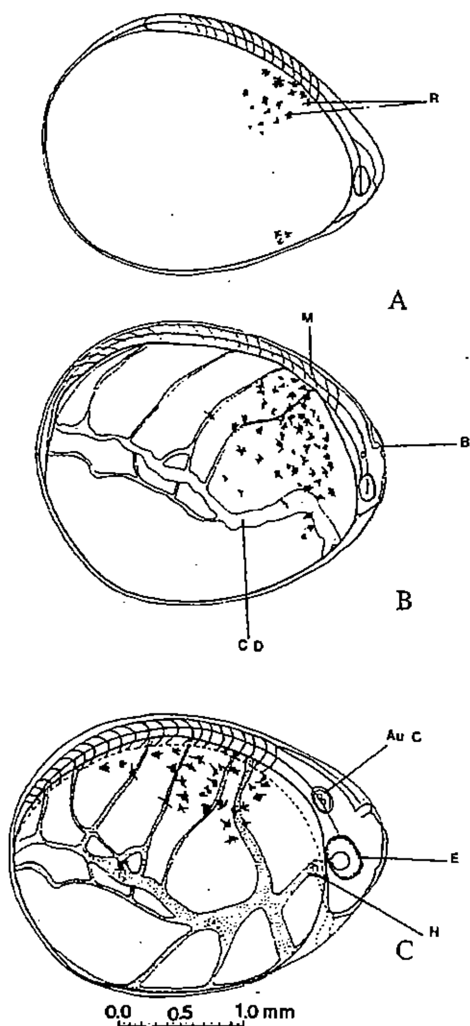
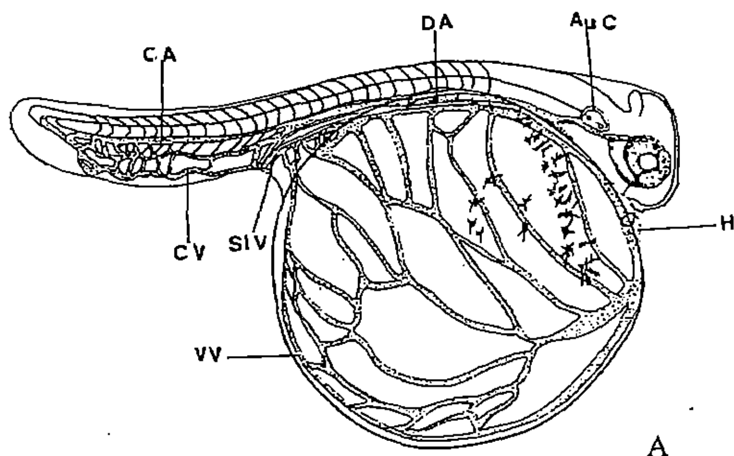


Plate II. Early stages of *Orechromis niloticus*

A- Age 36 hours from fertilization
B- Age 50 hours from fertilization
E- Age 68 hours from fertilization



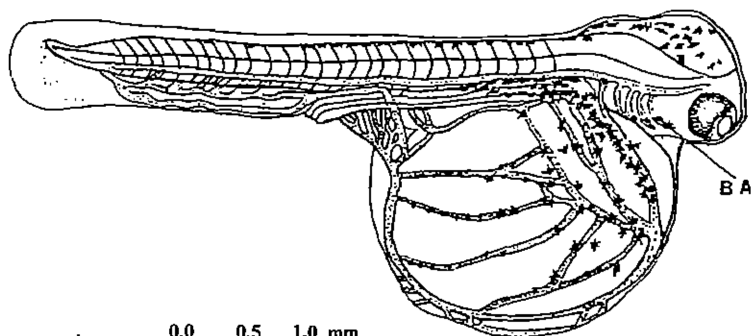
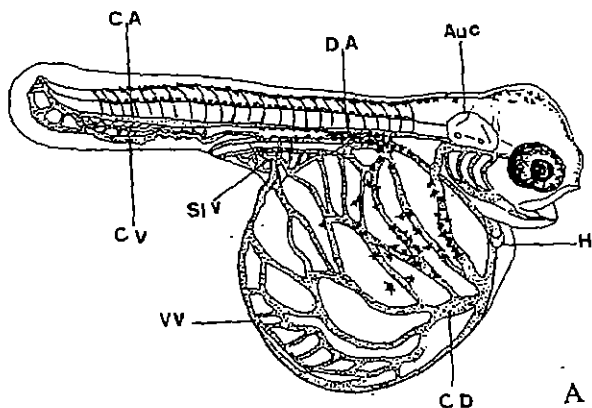


Plate III. Early stages of *Orechromis niloticus*

A- Newly hatched embryo
B- During the first day after hatching



0.0 0.5 1.0 mm

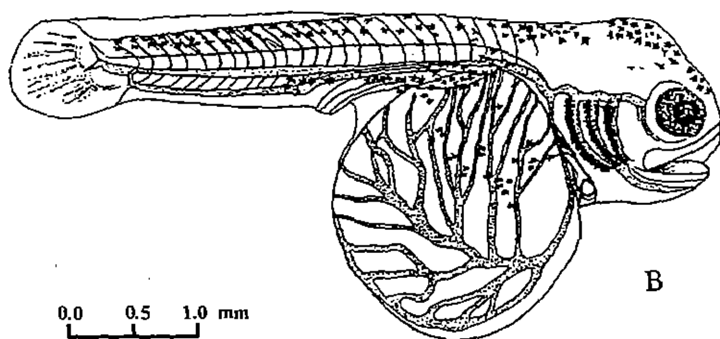


Plate IV. Early stages of *Orechromis niloticus*

A- About one day after hatching (age 110 hours from fertilization).
B-Age 138 hours from fertilization.

DIRECT EVIDENCE OF PHYSOSTOMOUS GAS BLADDER INFLATION IN PHYSOCLISTOUS FISH LARVAE

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Abstract

In situ and *in vivo* microcinematography show how walleye (*Stizostedion vitreum*) and striped bass (*Morone saxatilis*) larvae penetrate the air-water interface and use muscular contractions to place abdominal microbubbles into the pneumatic duct. These observations support putative evidence of physostomous gas bladder inflation in larval physoclistous species.

Introduction

Physostomous gas bladder inflation (GBI) physiology involves ingestion of air from the water surface and passage of the air through a pneumatic duct into the gas bladder. This mechanism represents the earliest evolution of the gas bladder from the lung and it is found only in more ancestral bony fishes such as salmonids, cyprinids, and catfishes. Physoclistous GBI physiology allows fishes to remove dissolved gases from the blood with a *rete mirablile* to inflate the gas bladder. This mechanism, found in the more derived fishes such as the percomorphs, allows fishes to regulate the volume of gas in the gas bladder without access to air from the water surface.

There is evidence of physostomous physiology in the larval stages of some fish species which are physoclistic at adults. Histological studies have identified the presence of a pneumatic duct in the larvae of several physoclistous percomorphs including sunfishes (*Lepomis cyanellus* and *L. macrochirus*), striped bass (*Morone saxatilis*), and walleye (*Stizostedion vitreum*) (Duwe, 1955; Doroshov et al., 1987; Marty et al., 1996). But whether or not this anatomical feature is a true ontogenetic recapitulation of phylogeny or a rudimentary structure has not been fully demonstrated. Denial of surface access during the larval period has been shown to preclude GBI development in walleye (Kindschi and MacConnell, 1989) and striped bass (Chapman et al., 1988), as well as other physoclists including the stickleback (*Gasterosteus aculeatus*) (von Ledebur, 1928, cited by Tait, 1960) and the bream (*Sparus auratus*) (Chatain and Ounais-Guschemann, 1990). Larvae of some more derived percomorphs including *Hemichromis bimaculata* and *Tilapia mossambica* (Cichlidae) have, however, been found to lack a pneumatic duct during the larval phase and to inflate their gas bladders without surface access (McEwen, 1940; Doroshov and Cornacchia, 1979).

It seems that if initial larval inflation does not occur in those species with a presumed physostomous larval phase, then the gas bladder degenerates, and those individuals lack hydrostatic balance. Fish without inflated gas bladders have been shown to have decreased growth and survival (Chatain, 1987), increased spinal deformities (Kitajima et al., 1994), and are unusable for stocking purposes (Kindschi and Barrows, 1993). Problems with GBI development resulting from poor larval performance has been an obstacle to larviculture of many species including the sea bass (*Macquaria novemaculeata*) (Battaglene and Talbot, 1990), bream (Chatain and Ounais-Guschemann, 1990),

striped bass (Doroshov et al., 1987), and walleye (Colesante et al., 1986). Techniques studied to improve larval GBI in these species include tank design, lighting, feeding technique, hormonal therapy, pH manipulation, water inflow method, salinity, and aeration, blowers or oxygens supersaturation (Cornacchia and Colt, 1984; Barrows et al., 1988; Brown et al., 1988; Chatain and Ounais-Guschemann, 1989; Bushman, 1992; Barrows et al., 1993; Moore et al., 1994). Those techniques that have seemed to enhance access through the surface-water interface have been the most successful in improved GBI performance (Chatain and Ounais-Guschemann, 1990; Summerfelt, 1991; Moore et al., 1994).

Histological studies of gas bladder anatomy and empirical studies of environmental influences on GBI have provided substantial indirect evidence of the occurrence and importance of physostomous GBI in physoclistous fish larvae. However, no verified observations of the larvae gulping air from the water surface, nor subsequently observed passage of air bubbles through a pneumatic duct into the gas bladder have been reported. What I offer here are recorded visual observations that larval walleye and striped bass penetrate the air-water interface, have air bubbles in the gut cavity during the initial GBI phase, and pass small bubbles from the gut cavity through the pneumatic duct. These observations were not the direct focus of any particular study, but were incidental to various studies of striped bass and walleye larvae for other purposes.

Methods and Materials

Observations were conducted during several years of studying larval striped bass and walleye in laboratory culture environments. Empirical studies of striped bass larvae at Aquatic Systems, Inc. in San Diego were conducted with a National Science Foundation research grant. Striped bass larvae were reared in 1,000-liter and 20-liter tanks in recirculating water culture systems. During this study, hundreds of thousands of striped bass larvae were reared in various conditions including using varying amounts of aeration, salinity gradients, and with different larval feeds. Thousands of larvae were anesthetized with Finquil (tricaine methane sulfonate) and examined through a dissecting scope to record gas bladder development, growth, and feeding performance, as well as any obvious anatomical abnormalities.

When air bubbles were first noticed in the guts of some of the striped bass specimens, it was perceived as an anomaly, perhaps related to slight gas supersaturation or conditions specific to the rearing environment. Further studies continued to reveal the presence of specimens with these 'gut bubbles' from various rearing conditions. It was then realized that these gut bubbles were perhaps part of the physostomous process hypothesized as a possible mechanism of initial GBI in striped bass by Doroshov et al. (1987). Direct evidence of the actual ingestion of air by striped bass larvae had, however, not been reported. We therefore affixed photographic equipment to the dissecting scope to record examples of gut bubbles in further observations. The occurrence of gut bubbles was also recorded and numerically related to rearing conditions. The results of those studies, including numerous examples of gut bubbles, are provided in an unpublished administrative report to the National Science Foundation.

Following the striped bass studies in San Diego, I conducted research at Iowa State University with high resolution microcinematography to obtain *in situ* observations of larval walleye. A larval culture laboratory was constructed specifically to enhance microcinemagraphic observations while conducting various studies of the influences of culture environments on larval walleye behavior and development. Cinematograpy equipment included a Sony CCD color video camera equipped with a high magnification zoom microscopic lens. Observations were recorded with a Sony Beta VCR which provides high resolution recording with various replay options to enhance observations an analyses of recorded information. A Zeiss SEM-(IBAS; 16 bit) image analysis system was used to produce photographic prints from selected video frames to represent descriptive aspects of larval activities. Slow motion, reverse, digital time display, and stop motion capabilities of the VCR

allowed the activities of each larvae in each aquaria to be analyzed from sample observations. Groups of 50 to 150 larvae were reared and observed in 3-liter aquaria for observations of larger populations. Groups of 10 to 20 larvae were occasionally removed from the larger aquaria and observed in 100-ml aquaria designed for high magnification microcinematography; and also occasionally, small numbers of larvae were removed from the rearing containers, anesthetized with Finquil, and *in vivo* observations were conducted through a dissecting scope.

Results and Discussion

Observations of striped bass larvae provided numerous *in vivo* micrographic and video recordings showing air bubbles in various positions and locations within the abdominal cavity. Although most specimens only contained one or few gut bubbles, many specimens had numerous bubbles (Figure 1). Although gut bubbles were generally observed in less than 5 percent of specimens examined, they were only observed during the period of development when initial GBI was occurring in the populations (i.e., from day-5 to day-10 posthatch) and were observed at higher rates of occurrence in studies with higher GBI rates.

Figure 1. A 6-mm, 8-day posthatch, striped bass larva showing air bubbles within the gut. The gas bladder (arrow) is inflated.

Doroshov et al. (1987) had shown the location of the pneumatic duct of larval striped bass to be anterior to the gas bladder, and positioned to transfer air from the gut lumen to an opening at an anterior-dorsal location on the gas bladder. The diameter of the pneumatic duct, however, seemed to be less than 20 µm, and most of the gut bubbles observed with lower magnification were larger than 100 µm. Scrutiny of some of the larvae at higher magnifications, however, revealed the presence of very small air bubbles alongside the larger bubbles in the larvae's guts (Figure 2).

The smallest bubbles seen in this higher magnification micrograph of the specimen shown in Figure 1 are approximately 15 µm in diameter. Of particular interest is the presence of at least two, or three, microbubbles that seem to be within the pneumatic duct; the position of those bubbles occur exactly within the alignment of the pneumatic duct as shown in Doroshov et al. (1987) and the more obscured appearance of those bubbles also indicates that they may be within tissues of greater density than those seen in the gut lumen.

Figure 2. The same larva shown in Figure1 showing, at higher magnification, details of air bubbles near the gas bladder.

The presence of these 15 µm microbubbles seems to indicate that the larval gut contained a surfactant. Small bubbles are unstable because they contract due to surface tension, or dissolve due to diffusion of gasses out of the bubble. Surfactants overcome this instability by reducing surface tension to allow formation and retention of various sized contiguous bubbles as we observed in the guts of the striped bass larvae. A surfactant may be provided in the larval striped bass to allow the microbubbles to form and maintain their size for transport through the 20 µm diameter pneumatic duct. This surfactant could be derived from larval bile or pancreatic secretions.

With visual evidence that gut bubbles were small enough to enter, and were seemingly present within, the pneumatic duct, our observations focused on what force was used to move the bubbles into and through the duct into a gas bladder. Fänge (1976) reported that gulped air is forced through the pneumatic duct in physostomes by a buccal force mechanism. Microcinematography of striped bass larvae provided insights into how microbubble may be forced into, and transported, through the pneumatic duct into the gas bladder.

In many of the specimens, we observed retrograde abdominal contractions occurring which moved the gut bubbles around within the gut, often squeezing the bubbles into elongated shapes, and often pushing the bubbles against the ventral edge of the gas bladder and, not only continually passed the bubbles along and against where the pneumatic duct would seem to open into the abdominal lumen, but also pushing with sufficient in force to deform the gas bladder shape, and move air bubbles already existing within the gas bladder (Figure 3). In this sequence of photographs, shown in order of occurrence from top to bottom, the large gut bubble is moved from a posterior position, behind the inflated gas bladder, into a position just below the anterior end of the gas bladder. As the retrograde movement occurs, the air bubble located within the gas bladder moves from an anterior to a more posterior position in response to the pressure of the gut bubble against the gas bladder.

Figure 3. Three micrographs, taken in rapid sequence, showing gut bubble movement in a striped bass larva.

Although it is possible that microbubbles, when present, could be forced into the pneumatic duct by buccal pressures alone, we hypothesized that the retrograde gut bubble movements against the ventral edge of the gas bladder could be creating differences in internal gas bladder pressure much like in the operation of bellows. That is, as the abdominal contractions pushed a larger bubble against the gas bladder, any fluid in the gas bladder might be expelled into the abdominal cavity through the pneumatic duct. As the large bubble then moves further and the pressure against the gas bladder is removed, the reformation of the gas bladder's original shape would create a lowered pressure within the gas bladder lumen and cause a reverse flow of fluid through the pneumatic duct. If, under this scenario, microbubbles were present against the opening of the pneumatic duct, these microbubbles would enter the pneumatic duct when the suction coincided with this placement. If the pneumatic duct contained any mechanism for one-way passage of bubbles (e.g., cilia or ridges) then the bubbles would pass through the duct and into the gas bladder as the bellows effect continued.

The anatomy of the developing larvae seems to assist in placement of gut bubbles against the ventral edge of the developing gas bladder. As seen in Figures 1 and 3, there is a fortuitous 'saddle-shaped' depression in the dorsal region of the oil globule in a larval striped bass. This saddle begins to form almost simultaneously to the expansion of the developing gas bladder (Figure 4); this micrograph shows the gas bladder in an early stage of dilation in a 4-day posthatch specimen with a 'saddle-shaped' depression forming directly below it in the oil globule.

Figure 4. Micrograph of the middle portion of a 4-day posthatch striped bass showing the uninflated, but dilated, gas bladder (arrow) just above a 'saddle' in the oil globule.

The observations of gut bubbles in striped bass larvae provided strong evidence of air gulping to perform physostomous GBI in a larval physoclist. Previous observations had indicated that the air was ingested at the air-water interface (Kindschi and MacConnell, 1989; Chapman et al., 1988). There were, however, no reports of actual observations that the surface tension was penetrated by a larval fish. Microcinemagraphic observations of larval walleye showed the larvae bouncing off the underside of the surface tension as if it were as solid as the aquaria sides. As Usinger (1956) stated, in reference to aquatic insects, 'to an organism of small size, this air-water interface can be an impenetrable barrier, a surface on which to rest, or a ceiling from which to hang suspended.' During review of hundreds of hours of cinematography of walleye larvae, the larvae were almost always seen 'bouncing' off, or in many instances, as Usinger noted, 'hanging suspended' from the underside of the surface tension.

During my striped bass and earlier walleye studies, I had noted that most often the walleye larvae were positioned against the tank edge. The microcinemagraphic view of larval walleye against the tank edge at the water surface showed that the larvae's heads fit fully into the meniscus of the water-edge interface (Figure 5). The point of contact of the larvae with the air-water interface is not with the snout, where the swimming force would be directed, but with the nuchal region. From this position, it seemed very difficult for the larvae to penetrate the surface tension barrier.

Figure 5. Micrograph from a video of a 12-mm, 8-day posthatch walleye larva in the meniscus (arrow) of an aquaria.

In the earliest studies, the focus of cinematography had been mostly at the tank edge water surface position because this was the location of most of the larvae and thus seemed to provide the best potential view of a larvae penetrating the surface to gulp air. But after examining the relationship of the meniscus to the larvae as shown above (Figure 5) it seemed that the penetration may be more likely when larvae were not against the tank edge. This realization changed the study focus.

Previous studies had associated illumination and tank color with improved performance of fish larvae. Nickum (1978) reported that walleye fry were so attracted to the sides of light-colored tanks that they ignored all forms of feed, whereas fry in dark-colored tanks were more evenly distributed and fed better. Ostrowski (1989) found that overall survival of dolphin (*Hippurus coyphaena*) averaged 50% in black tanks and 25% in uncolored (tan) tanks. Malison and Held (1991) reported that using internal tank lighting improved habituation of yellow perch (*Perca flavascens*) to prepared feed. None of these authors evaluated GBI although they all suggested better feeding resulted from either improved distribution or visual factors. Following review of these reports and the observations noted above on larval walleye behavior at the water surface as related to their ability to perform surface tension penetration for physostomous GBI, I began to study the effects of illumination on larval behavior.

Bristow and Summerfelt, 1994 showed that turbid water, induced with the addition of clay to the culture water, significantly improved growth and GBI performance in larval walleye culture. Simultaneous to that study, I investigated the effects of turbity on larval walleye behavior and found that turbidity altered the behavior of larval walleye in the rearing container (Rieger, 1995). In turbid water, compared to clear water, the larvae were less 'edge oriented.' This effect was attributed to the larvae's phototactic attraction to the source of light, which in clear water is the reflection of incoming light from the tank edge, even if the tank is painted black; whereas, in turbid water, reflective scattering of the incoming light precludes light reflection from the tank edge and brightens

the water column and eliminated attraction of the larvae to the tank edge. This effect caused significant changes in larval performance; they swim faster, feed earlier and GBI was nearly 100 percent, compared to about 50 percent in comparable clear water conditions.

Following the use of turbidity in the cinematography culture system, specimens were more easily observed away from the tank edge. Microcinematography eventually recorded a larva that seemed to exhibit a blurred movement unlike a 'bounce' off the surface tension. Replay of this event in slow motion confirmed that the larva had accomplished surface tension penetration. This discovery was not made until the sequence was viewed in slow motion (i.e., ¼ actual speed). This cinematography contained the only example of surface penetration recorded, but frame by frame analysis demonstrates the event very clearly (Figure 6).

The sequence of frames presented here show the larva as it prepares to penetrate the surface tension (A), as it pushes against the surface tension barrier (B), as it penetrates the barrier (C), and following penetration (D). The reflection of the larva from the underside of the surface tension is clear in frame A. A rapid motion of the caudal region, seen in the still micrograph as a blurred shadow, demonstrates the force the larva is exerting to achieve penetration (frames B & C). The clarity of the caudal region in segment D indicates that once penetrated, the surface tension suspends the larva's snout out of the water. The mouth of the larva, while closed prior to penetration, is open in frame D. The entire event shown in frames A through D occurs in ½ second. The brevity of the event explains the difficulty in obtaining a recording of this phenomenon. The meniscus of the nearby tank edge can be seen just to the right of the larva in these micrographs.

Figure 6. A sequence of video frames of a 12-mm, 8-day posthatch, walleye larva showing penetration of the water surface.

The observations shown and described above may offer fundamental knowledge of the mechanism of physostomous GBI in larval physoclists and are examples of the value of high resolution microcinematography to investigate dynamic microscopic biological phenomenon. Digitized recordings with various play back and image analyses options provide demonstrative exhibits of behavioral and physiological mechanisms of value to applied environmental physiology of fishes. I have found no other studies that visually demonstrate any other physostomous activity for a physoclistous species. These micrographs seem to be the first to show surface tension penetration, and the presence of gas bubbles within the pneumatic duct of, physoclist fish larvae. Studies conducted during, and subsequent to, these observations demonstrated the importance of the influences of the rearing environment on larval fish behavior and the effects of behavior on developmental performance (Rieger, 1995).

References

Barrows, F.T., W.A. Lellis, and J.G. Nickum. 1988. Intensive culture of larval walleyes with dry and formulated feed: note on swimbladder inflation. Prog. Fish-Cult. 50: 160-166.

Barrows, F.T., R.E. Zitzow, and G.A. Kindschi. 1993. Effects of surface water spray, diet, and phase feeding on swim bladder inflation, survival, and cost of production of intensively reared walleyes. Prog. Fish-Cult. 55: 224-228.

Battaglene, S.C., and R.B. Talbot. 1990. Initial swim bladder inflation in intensively reared Australian bass larvae, *Macquaria novemaculeata*. Aquaculture 86: 431-442.

Bristow, B.T., and R.C. Summerfelt. 1994. Performance of larval walleye cultured intensively in clear and turbid water. J. World Aquacult. Soc. 25: 454-464.

Brown, C.L., S.I. Doroshov, J.M. Nunez, C.G. Hadley, J. Vaneenennaam, R.S. Nishioka, and H.A. Bern. 1988. Maternal triiodothyronine injections cause increase in swimbladder inflation and survival rates in larval striped bass, *Morone saxatilis*. J. Exp. Zool. 248: 168-176.

Bushman, R.P. 1992. The effects of pH on gas bladder inflation of larval walleye. MS Thesis. ISU, Ames.

Chapman, D.C., W.A. Hubert, and U.T. Jackson. 1988. Influence of access to air and of salinity on gas bladder inflation in striped bass. Prog. Fish-Cult. 50: 23-27.

Chatain, B. 1987. The swimbladder in *Dicentrarchus labrax* and *Sparus auratus*. II. Influence of developmental anomalies on larval growth. Aquaculture 65: 175-181.

Chatain, B., and D.N. Ounais-Guschemann. 1990. Improved rate of initial swim bladder inflation in intensively reared *Sparus auratus*. Aquaculture 84: 345-353.

Colesante, R.T., N.B. Youmans, and B. Ziolkowki. 1986. Intensive culture of walleye fry with live food and formulated diets. Prog. Fish-Cult. 48: 33-37.

Cornacchia, J.W., and J.E. Colt. 1984. The effects of dissolved gas supersaturation on larval striped bass, *Morone saxatilis* (Walbaum). J. Fish Diseases 7: 15-27.

Doroshov, S.I., and J.W. Cornacchia. 1979. Initial swimbladder inflation in the larvae of *Tilapia mossambica* (Peters) and *Morone saxatilis* (Walbaum). Aquaculture 16: 57-66.

Doroshov, S.I., J.W. Cornacchia, and C. Hadley. 1987. Development of embryonic swimbladder in striped bass, *Morone saxatilis*. Pg. 3-20 in S.I. Doroshov and J.Van Eenennaam, eds. Swimbladder inflation in larval striped bass. Cal. Dept. F&G Res. Proj. #2-126-428-3; Univ. of California, Davis.

Duwe, A.E. 1955. The development of the gas bladder in the green sunfish. Copeia 2: 92-95.

Fänge, R. 1976. Gas exchange in the swimbladder. Pages 189-211 in G.M. Hughes, ed. Respiration of amphibious vertebrates. London: Academic Press.

Kindschi, G.A., and E. MacConnell. 1989. Factors influencing early mortality of walleye fry reared intensively. Prog. Fish-Cult. 51: 220-225.

Kindschi, G.A., and F.T. Barrows. 1993. Survey of swim bladder inflation in walleyes reared in hatchery production ponds. Prog. Fish-Cult. 55: 220-226.

Kitajima, C., Y. Tsukashima, S. Fujita, T. Watanabe, and Y. Yone. 1981. Relationship between uninflated swim bladders and lordotic deformity in hatchery-reared red sea bream, *Pagrus major*. Bull. Japanese Soc. Scientific Fisheries 47: 1289-1294.

Malison, J.A., and J.A. Held. 1991. Effects of fish size at harvest, initial stocking density and tank lighting conditions on the habituation of pond-reared yellow perch (*Perca flavescens*) to intensive culture conditions. Aquaculture 104: 67-78.

Marty, G.D., D.E. Hinton, and R.C. Summerfelt. 1995. Histopathology of swimbladder noninflation in walleye (*Stizostedion vitreum*) larvae: role of development and inflammation. Aquaculture 133:

McEwen, R.S. 1940. The early development of the swim bladder and certain adjacent parts in *Hemichromis bimaculata*. J. Morphol. 67: 1-57.

Moore, A., M.A. Prange, R.C. Summerfelt, and R.P. Bushman. 1994. Evaluation of tank shape and a surface spray for intensive culture of larval walleyes fed formulated feed. Prog. Fish-Cult. 56: 100-110.

Nickum, J.G. 1978. Intensive culture of walleyes: The state of the art. AFS Special Pub. 11: 187-194.

Ostrowski, A.C. 1989. Effect of rearing tank background color on early survival of dolphin larvae. Prog. Fish-Cult. 51: 161-163.

Rieger, 1995. Behavior of Larval Walleye. Ph.D. Dissertation, Iowa State University, Ames.

Summerfelt, R.C. 1991. Non-inflation of the gas bladder of larval walleye (*Stizostedion vitreum*): Experimental evidence for alternative hypotheses of its etiology. Pages 290-293 in P. Larens, P. Sorgeloos, E. Jaspers, and F. Ollevier, eds. Larvi' 91 - Fish and Crustacean Larviculture Symposium. Eur. Aquaculture Society, Special Pub. No. 15, Gent, Belgium.

Tait, J.S. 1960. The first filling of the swim bladder in salmonoids. Can. J. Zool. 38: 179-187.

Usinger, R.L., editor. 1956. Aquatic Insects of California. Univ. of California Press. Berkeley.

Von Ledebur, J.F. 1928. Beiträge zur physiologie der schwimmblase der fische.I.Z.vergleich Phys 8:445-460.

EMBRYONIC AND LARVAL DEVELOPMENT OF

PUNTIUS SCHWANENFELDII (BLEEKER)

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Abstract

Embryonic and larval development of *Puntius schwanenfeldii* (Bleeker) were described under laboratory condition. Eggs were obtained through induced breeding using acetone-dried carp pituitary hormone at 6mg/kg body weight. The fertilized eggs had an average diameter of 1.02 ± 0.03 mm, non-adhesive, demersal and were covered with a thin layer of membrane. The 2-cell egg stage was first observed after 20 minutes of fertilization and by 6hr 20 min the head and tail were noted. Hatching occurred at 16 hrs at 25 C and the length of the newly hatched larvae were 1.96 ± 0.03 mm. Larvae were inactive for most of the time and lay at the bottom of the tank. Organogenesis was completed 30 days after hatching and by this time the juvenile resembled the adults.

Introduction

Puntius schwanenfeldii (Bleeker) is a cyprinid and is widely distributed throughout Thailand, Malaysia, Sumatera and Borneo (Smith, 1945). The species has commercial importance for it is used as food and aquarium fish. Studies pertaining to aspects of reproduction (Harmin and Gintua, 1995; Harmin et al., 1994;1993) and genetics (Siraj et al., 1994) have been conducted, however; information with regards to the early life development of the fish is not documented. Thus, our objective is to describe the early egg and larval development of *Puntius schwanenfeldii* as a basis for future physiological studies.

Materials and Methods

The male and female fish weighing between 130 to 250 g were used and maintained in earthen pond and fed with pelleted feed for approximately a year. Three trials of induced spawning were attempted to produce the larvae, however; only the last trial was used to describe the egg and larval development. A single injection of carp pituitary extract was administered to the females at the rate 6 mg/kg body weight (bw), whereas the males received 3 mg/kg bw to increase spermiation. The eggs were fertilized following the dry method procedure and incubated at water temperature of 25 ± 2 C.

Observation on the embryonic development were based on samples (n=6-7) taken prior to fertilization, and at 5, 10, 15, 30, 60 and 120 post-fertilization until hatching. Larvae were sampled on day 0, 1, 2, 3, 4, 5, 7, 10, 14, 21, 30, 40 50 and 60. Developmental stages were observed using microscope and profile projector. Samples were preserved in 10% buffered formalin.

Results and Discussion

Unfertilized eggs measured 0.91 ± 0.004 in diameter. Eggs were spherical, greenish yellow in color and non adhesive. First cell cleavage occurred 20 min. after fertilization followed by second cleavage within 30 min after fertilization. Egg diameter increased to 1.30 ± 0.009 mm. and the cell continued to divide rapidly after eight blastomere stage. Morula and blastula stage occurred at 100 min and 180 min, respectively. By 225 min. gastrula appeared and it was observed that there was no significant increased in the egg diameter at this stage. The head and tail region began to appear after 6 hr. 20 min post-fertilization. At 8 hr. the eye vesicle could be observed. The embryo increased in size and somites were visible and followed by the notochord at 10 hr post fertilization.

Hatching began between 12 and 16 hr after fertilization. The hatchlings were transparent and non pigmented. The average length of the larvae were 1.96 ± 0.03 mm. One day old larvae were observed to be still transparent with heart beat between 160-170 per min. The larvae increased in size and the average length measured approximately 3.70 ± 0.03 mm. The number of myomeres observed was between 29 and 31.

On the second day, the larvae appeared to be more active. However, they tended to lay at the bottom of the tank for most of the time, with size increment up to 3.82 ± 0.05 mm. Anal structure was observed and located between the 20th and 21st myomeres. Three-day old larvae measured 4.12 ± 0.02 mm in total length, and began its first exogenous feeding eventhough the yolk sac have not been completely resorbed.

One-week old larvae were very active though still transparent with observable myomeres. Egg yolk was completely resorbed. Melanophores were observed to develop in the head region. By day 10, the larvae were free swimming with average body length increased to 6.30 ± 0.16 mm. and fed entirely on exogenous food.

Melanophores were found all over the head of the two-week old larvae with body length increased to about 8.34 ± 0.02 mm and an inferior mouth was clearly observed. By the third week, the larvae increased in size to about 12.24 ± 0.03 mm. and by one month the total length of the larvae averaged to 15.6 ± 0.02mm. The body was not transparent and myomeres were not observable any more due to the appearance of some scales on the body. After 2 month, the body length of the fish was 30.65 ± 0.06 mm, weighing 0.26 ± 0.01 g. The body were covered completely with scales and all fin development have been completed. Larvae were more active and swam in groups.

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References

Harmin, S.A. and Gintua, D. 1995. Use of gonadotropin-releasing hormone analogs in accelerating oocyte development, inducing ovulation and spawning of cultured fishes. *In* "Proceedings of the First National Symposium on Biomedical Sciences," 20 - 21 March 1995, Kuala Lumpur, Malaysia *(in press)*

Harmin, S.A, R. Joannes, and Cheah, S.H. 1994. Influence of dietary protein level on growth and ovarian development in lampam sungai, *Puntius schwanenfeldii* (Bleeker), "Proceedings of the Malaysian Science and Technology Congress '94," pp. 155-159. Confederation of Scientific and Technological Associations in Malaysia (COSTAM), Kuala Lumpur, Malaysia.

Harmin, S.A., R. Joannes, and Cheah, S.H. 1993. Effects of a single injection of carp pituitary extract and human chorionic gonadotropin on germinal vesicle migration and ovulation in lampam sungai, *Puntius schwanenfeldii* (bleeker): a preliminary study. *In.* "Proceedings of the National Seminar-Workshop on Breeding and Seed Production of Important Cultured Finfishes in the Philippines, SEAFDEC/AQD," 4-5 May 1993. Tigbaun, Iloilo, The Philippines *(in press)*.

Siraj, S.S., S.A. Harmin, and Patimah, I. 1994. Reproductive performance and genetic research in *Puntius* species at Universiti Pertanian Malaysia. *In* "Proceedings of the Malaysian Science and Technology Congress '94," pp. 160-166. Confederation of Scientific and Technological Associations in Malaysia (COSTAM), Kuala Lumpur, Malaysia.

Smith, H.M. 1945. The freshwater fishes of Siam or Thailand. United States Govt. Printing Press. 622 pp.

TEMPERATURE AND THE EFFICIENCY OF DEVELOPMENT DURING ENDOGENOUS FEEDING IN HERRING EMBRYOS AND YOLK SAC LARVAE.

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Abstract

Oxygen uptake, dry weights and energy contents of herring (*Clupea harengus*) were measured periodically from the time of egg fertilization to the end of the yolk sac stage (without added food). Temperature of incubation (3.5°C to 17°C) had little effect on efficiency of conversion of egg reserves to body material.

Introduction

The effects on fishes of changes in water temperature, due to inter-annual variation and to climate change, are likely to be greatest on the younger life stages (Blaxter, 1992). Optimum temperatures for larval stages of fish have been estimated based on hatching rates (eg. Rana, 1990a) and on upper and lower lethal temperatures (Kamler, 1992). Enzyme parameters and metabolic rates have also been used to define optimum development temperatures; thus enzyme K_ms show a minimum at the optimum temperature (Klyachko & Ozernyuk, 1991; Klyachko & Ozernyuk, 1994; Ozernyuk *et al.* 1994). Time-integrated oxygen consumption has a minimum over the optimum temperatures range and this minimum value can increase by as much as 100% at extremes of temperature (Zotin & Ozernyuk, 1966; Alexeeva & Ozernyuk, 1987).

Because of the metabolic costs of development at different temperatures, which are reflected by oxygen consumption values, it would be expected that larvae dry weights and heat contents (heats of combustion) would show a maximum over the optimum temperature range. Yolk utilization efficiencies based on dry weights and energy contents have been used to demonstrate temperature optima in Dover sole (*Solea solea*), (Flüchter & Pandian, 1968), California grunion (*Leuresthes tenuis*), (Ehrlich and Muszynski, 1982), yellowtail flounder, (*Limanda ferruginea*), (Howell, 1980), American plaice, (*Hippoglossoides platessoides*), (Howell & Caldwell, 1984), *Orechromis niloticus*, (Rana 1990b) and African catfish (*Clarias gariepinus*), (Kamler *et al*, 1994).

In the work reported here, dry weights, energy contents and time-integrated oxygen consumptions were measured as a function of temperature to test the hypothesis that an optimal temperature for development to hatch and to first feeding in herring (*Clupea harengus*) larvae could be determined from these measures of yolk conversion efficiency, and thus be of use in predicting the responses of different populations of herring to climate change.

Materials and Methods

Herring were caught by trawl at their spawning grounds in the Firth of Clyde, and ripe gonads were removed and placed on ice on board ship. Gonads were returned to the laboratory on ice, and within a few hours the eggs were spread thinly onto glass plates and fertilized with sperm from 3 or 4 males (Blaxter, 1968). Embryos and larvae were incubated at the following temperatures: 3.5, 5, 8, 15, and 17°C

For the purpose of calculation of total oxygen consumed, elapsed times from fertilization to hatch and from fertilization to exhaustion of the yolk sac were required.

Individual embryos or larvae were filtered onto pre-weighed discs of Whatman glass fibre filter paper type GF/C (which had been heated to 450°C overnight to destroy any combustible material), and washed well with distilled water to remove seawater salts. After drying in vaccuuo the discs were weighed and energy contents (heats of combustion) determined by micro bomb calorimetry (Scott & Marlow, 1982).

Oxygen uptake rate was determined by measuring the decrease in oxygen tension in a closed respirometer chamber. The oxygen content was determined by aspirating the sample with oxygen-free nitrogen and measurement of the oxygen in the gas stream with a cadmium fuel cell (Peck & Uglow, 1990). The uptake rate was determined from the linear portion of the plot of oxygen concentration versus time. (In the case of larvae the linear portion extended from saturation to 50-60% of saturation.)

The total oxygen consumed from fertilization to hatch or from fertilization to exhaustion of the yolk sack was determined by integration of the measured uptake rates with respect to time. For this purpose the uptake rates were assumed to be a linear function of time starting at zero, since the data did not justify a more complex model (for example see Figure 1). From regressions through the origin of the uptake rates on time, the slopes and 95% confidence intervals were calculated. In the case of measurements of progeny of a single fish at six temperatures it was not practical to measure the oxygen uptake of embryos as well as larvae and so the data for Figure 9 were calculated from the measurements of oxygen consumption of larvae only, using either the number of days from fertilization to hatch or from fertilization to exhaustion of the yolk sack as the integration limits.

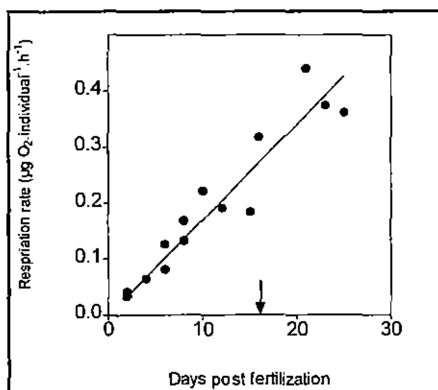

Fig. 1 Typical plot of respiration rate of developing embryos and yolk sac larvae versus number of days after fertilization; in this case Clyde herring at 8°C (with regression line through the origin). The arrow indicates hatch date.

Results

Dry weights and heats of combustion. The dry weights of newly hatched larvae and of larvae at the point of exhaustion of their yolk sacs showed a remarkable constancy over the temperature range studied, and this was reflected in the corresponding heats of combustion (albeit with greater errors), Figures 2 &3. The two batches of eggs chosen had weights not differing greatly from each other but curiously, the heavier batch produced the lighter progeny with corresponding lower efficiencies of egg conversion (Table I).

Source and cohort	Efficiency by weight	Efficiency by energy content	Biological zero T_0
Clyde (3.5-17°C) no 1	51.3 (hatch)	44.9 (hatch)	0.8°C (hatch)
	40.2 (exhaust)	36.2 (exhaust)	0.0°C (exhaust)
Clyde (3.5-17°C) no 78	74.7 (hatch)	76.3 (hatch)	
	53.0 (exhaust)	48.7 (exhaust)	

Table I. Efficiencies of conversion of egg to larva, and temperature of biological zero, T_0, calculated for times to hatch and to exhaustion of yolk sac.

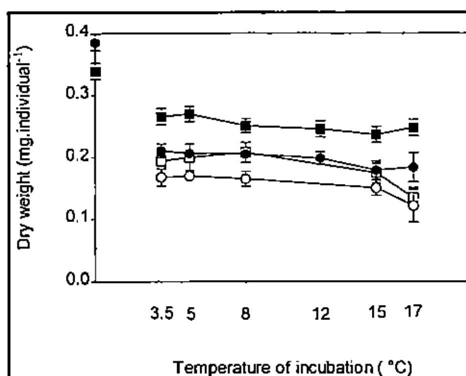

Fig. 2 Dry weights of unfertilized eggs (symbols on ordinate axis), of newly hatched larvae (solid symbols) and larvae at the point of exhaustion of their yolk-sacs (unfilled symbols). ● ○, Progeny of Clyde fish no. 1, and ■ □,progeny of fish no. 78. The centres of the symbols represent means of 10 individuals and the bars the SDs.

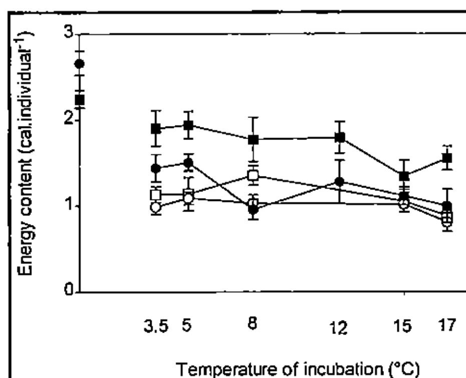

Fig. 3 Energy contents (heats of combustion) of unfertilized eggs (on ordinate axis) and of newly hatched larvae (solid symbols) and of larvae at the point of exhaustion of their yolk sacks (unfilled symbols). ● ○, Progeny of fish no 1, and ■ □, progeny of Clyde fish no. 78. The centres of the symbols represent means of 9 or 10 individuals and the bars the SDs. (1 calorie = 4.187 joules)

Oxygen consumption
Oxygen consumptions to hatch and to exhaustion of yolk sack stages showed little variation with temperature (Figure 4) and certainly not the U shaped response with high values at the extremes of temperature found by other investigators for a terrestrial fish (Alexeeva & Ozernyuk, 1987, Zotin & Ozernyuk , 1966). Indeed, the highest oxygen consumption found here was recorded for yolk-sack-exhausted larvae at 8°C - which is likely to be near the optimum temperature for the species.

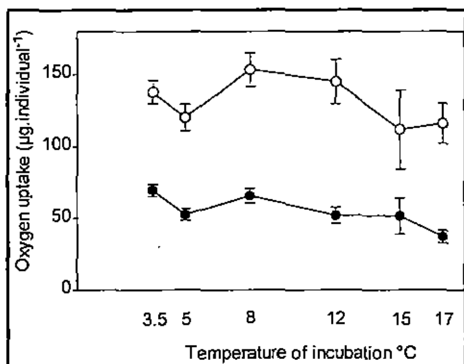

Fig. 4 Total oxygen consumed from fertilization to hatch, ●, and from fertilization to the point of exhaustion of yolk sack, O, for batches of larvae from Clyde fish no 1. Error bars represent 95% confidence intervals of the slopes of each regression (such as Fig. 1).

Discussion and Conclusion

Herring larvae were raised from the fertilized egg to the stage of exhaustion of the yolk sack over as wide a range of temperature as has been found practical in this laboratory, such that the extremes of temperature used (3.5°C and 17°C) must be close to the limits for herring. For a fish with a biological zero of 0°C, this represents a 4.9 fold temperature range. On the basis of previous work in other laboratories it was expected that plots of dry weights or energy contents versus temperature would show domed curves and plots of oxygen consumptions versus temperature would show U shaped curves. There was no such effect; therefore the original hypothesis that a region of greatest efficiency could be found and used to determine the optimal temperature range for herring stocks appears untenable. Examination of Figures 7, 8 & 9 suggest that there may be a slight decrease in dry weights, energy contents and oxygen uptakes for newly hatched Clyde herring larvae as the temperature increases. However the effect is small and, if real, is contradictory since a decrease in dry weight or energy content would imply an increase in oxygen consumption rather than a decrease. An increased loss of metabolites to the medium with temperature would be required to account for this.

It must be concluded that either the "fitness" of yolk sac larvae and larvae at the point of exhaustion of the yolk sac is approximately equal from 3.5°C to 17°C, or that fitness is determined by some parameter which does not impinge on efficiency of conversion of egg tissue to larval tissue. This then raises the question of what are the biochemical compensatory mechanisms which enable herring to achieve this constancy of efficiency, what are the biochemical constraints which finally determine the limits of temperature and at what developmental stage do they operate ? There are a few tantalizing clues in the literature regarding possible biochemical compensatory mechanisms. Different K_ms for the same enzyme isoform have been found for fish acclimated at different temperatures, which was attributed to differences

in tertiary structure (Klyachko & Ozernyuk, 1991; Klyachko & Ozernyuk, 1994; Ozernyuk *et al.* 1994). In addition different isoforms of the same myosin ATPase have been shown to be induced either by acclimation to different temperatures (Gerlach et al. 1990) or by rearing at different temperatures (Crockford & Johnston, 1993).

References

Alexeeva, TA & Ozernyuk, ND 1987 Energetical exchange and temperature optimum of loach development. *Zhurnal Obshchei Biologii* **48**, 525-531.

Blaxter, JHS 1968 Rearing herring larvae to metamorphosis and beyond. *Journal of the Marine Biological Association of the U.K.* **48**, 17-28.

Blaxter, JHS 1969 Development: eggs and larvae. *Fish Physiology* **3**, 177-252.

Blaxter, JHS 1992 The effect of temperature on larval fishes. *Netherlands Journal of Zoology.* **42**, 336-357.

Crockford, T and Johnston, IA 1993 Developmental changes in the composition of myfibrillar proteins in the swimming muscles of Atlantic herring, *Clupea harengus*. *Marine Biology* **115**, 15-22.

Ehrlich, KF, Muszynski, G 1982 Effects of temperature on interactions of physiological and behavioural capacities of larval California grunion: Adaptations to the planktonic environment. *Journal of Experimental Marine Biology and Ecology* **60**, 223-244.

Flüchter, J and Pandian, TJ 1968 Rate and efficiency of yolk utilization in developing eggs of the sole *Solea solea*. *Helogoländer wissenschaft Meersuntersuchen* **18**, 53-60.

Gerlach, G-F, Turay, L, Malik, KTA, Lida, J, Scutt, A and Goldspink, G 1990 Mechanisms of temperature acclimation in the carp: a molecular biology approach. *American Journal of Physiology* **259**, R237-R244.

Howell, WH 1980 Temperature effects on growth and yolk utilization in yellowtail flounder, *Limanda ferruginea*, yolk-sac larvae. *Fishery Bulletin* **78**, 731-739

Howell, WH and Caldwell, MA 1984 Influence of temperature on energy utilization and growth of embryonic and prolarval American plaice, *Hippoglossoides platessoides* (Fabricius). *Journal of Experimental Marine Biology and Ecology.* **79**, 173-189.

Kamler, E & Kato, T 1983 Efficiency of yolk utilization by *Salmo gairdneri* in relation to incubation temperature and egg size. *Polskie Archiwum Hydrobiologii.* **30**, 271-306.

Kamler, E 1992 Early life history of fish. An energetics approach. Chapman & Hall, London. 268 pp.

Kamler, E, Szlaminska, M, Kuczynski, M, Hamackova, J, Kouril, J and Dabrowski, R 1994 Temperature-induced changes of early development and yolk utilization in the African catfish *Clarias gariepinus*. *Journal of fish Biology.* **44**, 311-326.

Klyachko, OS and Ozernyuk. ND 1991 Temperature adaptations of metabolism: Effects of temperature on the kinetic properties of lactate dehydrogenase (Km) during development of various species of fish. *Doklady Akademii Nauk. SSSR.* **319,** 1252-1255.

Klyachko. OS and Ozernyuk, ND 1994 The effect of temperature on the kinetic properties of lactate dehydrogenase from embryos of various fish species *Comparative Biochemistry and Physiology* **107B**, 593-595.

Ozernyuk., ND, Klyachko, OS, Polosukhina, ES, Ermolaeva, LP and Ovsyannikova, OE 1994 Thermal Metabolic Adaptations in Poikilothermic animals at various stages of ontogenesis with special reference to carbohydrate metabolism enzymes. *Izvestiya Rossiiskoi Akademii Nauk, Seriya Biologicheskaya* 4, 519-527.

Peck, LS and Uglow, RF 1990 Two methods for the assessment of the oxygen content of small volumes of seawater. *Journal of Experimental marine Biology and Ecology* **141**, 53-62.

Rana, KJ 1990a Influence of incubation temperature on *Oreochromis niloticus* (L.) eggs and fry. I. Gross embryology, temperature tolerance and rates of embryonic development. *Aquaculture* **87**, 165-181.

Rana, KJ 1990b Influence of incubation temperature on *Oreochromis niloticus* (L.) eggs and fry. II. Survival, growth and feeding of fry developing soley on their yolk reserves. *Aquaculture* **87**, 183-195.

Scott, JM and Marlow, JA 1982 A microcalorimeter with a range of 0.1-1.0 calories. *Limnology and Oceanographgy* **27,** 585-590.

Zotin, AI and Ozernyuk, ND 1966 Effect of temperature on respiration and ATP level during cleavage of loach eggs. *Doklady Akademii Nauk SSSR* **171,** 1002-1004.

A SPECIALIZED ROLE FOR THE PACIFIC HERRING EGG CHORION IN SPERM MOTILITY INITIATION

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INTRODUCTION

The Pacific herring, *Clupea pallasi*, is a commercially and ecologically important marine teleost that spawns in bays and estuaries having freshwater input (Alderdice and Velsen, 1971). Like many other teleost fish, herring reproduce by broadcast spawning of sperm and eggs. Typically, males spawn first, and released sperm serves to attract females as well as other males to the spawning site (Stacey and Hourston, 1982; Carolsfeld and Sherwood, 1995). Herring eggs are extremely adhesive, and attach to a variety of solid substrates upon which the fertilized eggs undergo development through hatching. Herring spawning biomass in San Francisco Bay, which is the basis of a large commercial fishery, experienced a decline during the late 1980's and early 1990's (CDFG, 1992). During this period, salinities in the Bay increased dramatically due to drought conditions and diversion of freshwater runoff. This has led to the hypothesis that altered salinities may adversely affect herring reproduction.

It has long been recognized that the eggs of many invertebrates and vertebrates possess properties that enhance or activate sperm (reviewed by Morisawa 1994). For example, the jelly coat of sea urchin eggs induces the sperm acrosome reaction, a prerequisite for successful fertilization. Similarly, mammalian eggs possess factors that capacitate sperm and induce the acrosome reaction, as well as facilitate binding of the sperm to the egg plasma membrane. The plasma membrane of teleost eggs is surrounded by a thick chorion that is impenetrable to sperm except in the region of the micropyle, located at the animal pole. Fertilization occurs only when a motile sperm finds and traverses the micropylar canal to fuse with the egg plasma membrane. With the exception of the Japanese bitterling, in which the micropyle region has chemoattractant properties, the micropyle of most teleosts is considered to play a passive role, serving primarily as a barrier to the passage of supernumerary sperm and as a pathway for the fertilizing sperm.

The micropyle region of the eggs of Pacific herring (*Clupea pallasi*) has been shown to play an active role in the activation of herring sperm (Yanagimachi and Kanoh, 1953; Yanagimachi, 1957; Yanagimachi *et al.*, 1992; Pillai, *et al.*,1993; Griffin *et al.*, 1996). Unlike the sperm of most

organisms, herring sperm are virtually immotile at the time of spawning, however, contact with the micropyle region of the herring egg initiates sperm motility. Our laboratory has focused on the basic mechanisms of the above phenomenon during herring gamete interaction, as well as the role of environmental factors in modulating fertilization.

SPERM MOTILITY INITIATING FACTOR

Our laboratory has isolated and partially characterized a component of the herring egg shown to induce motility, termed sperm motility initiating factor, or SMIF (Pillai *et al.*, 1993; Griffin *et al.*, 1996). SMIF is a 105 kDa basic glycoprotein that is localized to the region surrounding the vestibule, which contains the micropylar opening. Evidence for sperm motility initiation by the micropyle region of the *C. pallasi* chorion was first observed by Yanagimachi and Kanoh (1953). Immotile sperm in contact with the micropyle region were observed to become motile, while sperm in contact with other regions of the chorion remained immotile. We have recently observed that the number of motile sperm in the micropyle region of eggs is significantly reduced in the presence of a polyclonal antibody generated against SMIF (Griffin, *et al.*, 1996). Similarly, fertilization rates of eggs incubated in antibody are also reduced. Immunostaining of anti-SMIF treated eggs confirmed a specialized role for SMIF at the site of sperm-chorion interaction; specifically, the region surrounding the vestibule which contains the micropylar opening (Fig. 1).

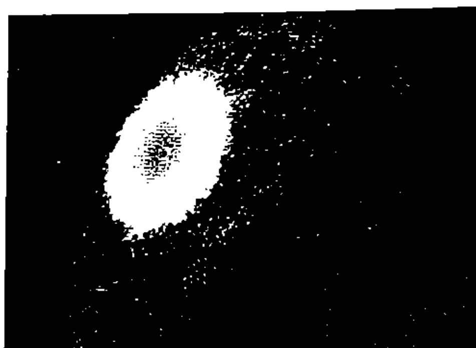
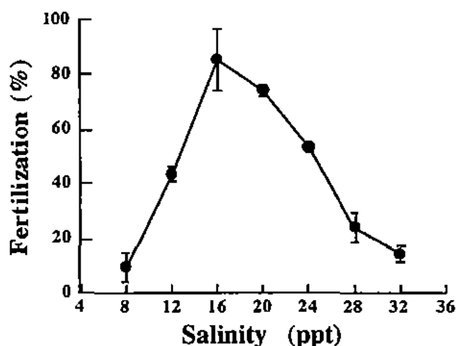


Figure 1. Anti-SMIF labeling of the herring chorion demonstrating staining in the micropylar region only. Intensity of staining increases from the micropylar opening towards the periphery of the micropyle.

SMIF appears to be a non-diffusible component of the micropyle, in that sperm motility eventually ceases if a spermatozoon swims away from and does not re-contact the micropyle. Furthermore, extensive washing in 1/2 sea water does not significantly reduce the motility initiating ability of chorions (Yanagimachi, 1957; Yanagimachi *et al.*, 1992). SMIF can, however, be removed from egg chorions using 1/2 sea water which is acidified to pH 3.5, and this "soluble" SMIF is a potent inducer of sperm motility *in vitro*. SMIF clearly differs from a family of small molecular weight peptides, termed herring sperm activating peptides (HSAP's), which have also been reported to enhance sperm motility (Morisawa *et al.*, 1992; Morisawa, 1994; Oda *et al.* 1995).

EFFECTS OF SALINITY ON FERTILIZATION AND SMIF-INDUCED SPERM MOTILITY

Our laboratory has been investigating the effects of salinity and specific ions on herring fertilization and early development in the San Francisco Bay population. It has previously been shown that the optimal salinity for both fertilization and development is approximately half-strength seawater (Yanagimachi, 1953; Yanagimachi and Kanoh, 1953; reviewed by Alderdice and Hourston, 1985). Our results for the San Francisco Bay population during the 1992-93 season confirm this (**Fig 2 A, B**). A role for specific ions is suggested by preliminary results indicating that increasing concentrations of sodium in 1/2 sea water decrease fertilization rates in a dose-dependent manner. The roles of other ions remain to be clarified.



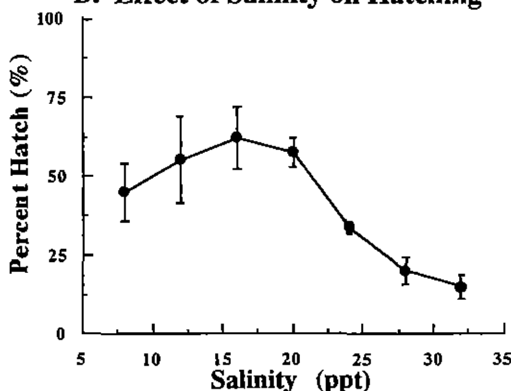


Figure 2 A,B. Graphs illustrating the effects of salinity (in ppt) on percent fertilization (A) and hatching success (B) in Pacific herring.

Alterations in salinity affect SMIF induced sperm motility *in vitro* as well, with very low or very high salinities reducing motility (Fig. 3). This led us to extend our initial investigations to include both the salinity and ionic requirements for initiation of herring sperm motility, as well as to further characterize the role of SMIF in inducing sperm motility (Yanagimachi *et al.*, 1992; Pillai *et al.*, 1993). Initiation of herring sperm motility is highly dependent on ionic conditions in the surrounding medium. Motility initiation in the micropyle region requires extracellular calcium and potassium, as evidenced by lack of SMIF induced motility in calcium or potassium free 1/2 strength artificial seawater (1/2 Ca^{++} or K^{+} FASW) (Yanagimachi *et al.*, 1992). However, manipulation of intracellular levels of these cations alone is not sufficient to trigger motility. Interestingly, vigorous motility can be initiated in 1/2 low sodium (2 mM) artificial seawater (1/2 Na^{+}FSW), in the absence of SMIF. The likelihood of herring sperm experiencing seawater lacking sodium is remote (the Na^{+} concentration in 1/2 sea water is approximately 220 mM), thus the physiological relevance of motility in low sodium media is unknown. Although Ca^{++} is required for SMIF-induced motility initiation, high concentrations of Ca^{++} or Mg^{++} can inactivate or remove SMIF from eggs (Yanagimachi and Kanoh, 1953; Yanagimachi, 1957).

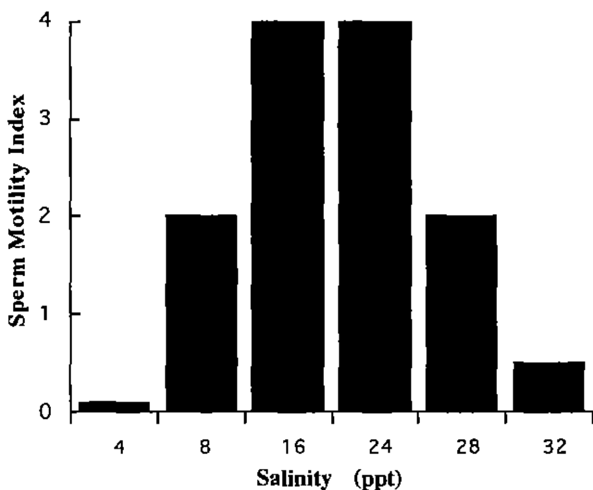


Figure 3. Graph illustrating the effect of varying salinities on SMIF induced herring sperm motility *in vitro*. Motility was qualitatively graded on a scale of 0 to 4; 0 = no motility; 4 = 4+ motility.

Since low sodium sea water induced sperm motility *in vitro*, we investigated a role for sodium in SMIF-induced activation. Amiloride, a sodium channel blocker, was found to inhibit SMIF-induced motility. In addition, elevated extracellular sodium inhibited SMIF-induced motility in a dose dependent manner, similar to the one observed for fertilization. Using the fluorescent sodium indicator Sodium Green, we have recently found a decrease in intracellular sodium upon

SMIF activation of sperm (not shown). This finding is consistent with the hypothesis that there is an efflux of sodium from *C. pallasi* sperm following interaction with SMIF, and that elevated salinities (such as those experienced during the extended drought of the late 1980s) at the time of spawning may adversely affect the ability of sperm to initiate motility.

CONCLUSIONS

The role of SMIF in initiating herring sperm motility may reflect the spawning behavior of herring. Most fish sperm have a limited, very short lifespan once released and diluted into the aqueous environment (reviewed by Morisawa, 1994). Since male herring typically spawn prior to females, it may be advantageous for the sperm to remain immotile, conserving metabolic energy until eggs are actually present in the vicinity of the sperm. Interaction of the sperm with the chorion factor SMIF, possibly through a receptor mediated mechanism, may trigger processes that initiate motility. The ability of SMIF to activate sperm appears to be dependent upon a reduction in salinity of the surrounding medium, with sodium potentially playing a key role in this regulation. Other ions such as calcium and potassium undoubtedly also have roles, perhaps facilitating SMIF binding to a sperm receptor or acting as counter ions during sodium efflux. Our current research efforts involve these and other related issues.

ACKNOWLEDGEMENTS

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REFERENCES

Alderdice, D.F., and A.S. Hourston. 1985. Factors influencing development and survival of Pacific herring (*Clupea harengus pallasi*) eggs and larvae to beginning of exogenous feeding. Can. J. Fish. Aquat. 42:56-68.

Alderdice, D.F., and F.P. J Velsen . 1971. Some effects of salinity and temperature on early development of Pacific herring (*Clupea pallasi*). J. Fish Res. Board Canada, 28:1545-1562.

California Department of Fish and Game. 1992. Exhibit WRINT-DFG-Exhibit No. 6. Water Quality-Water Rights Proceedings on the San Francisco Bay/Sacramento-San Joaquin Delta. 28.

Carolsfeld, J., and N. M. Sherwood. 1994. Characterisation of the response to spawning pheromone in Pacific herring. Proceedings of the Seventh Pacific Coast Herring Workshop. pp. 55-56.

Griffin, F.J., C.A. Vines, M. C. Pillai, R. Yanagimachi, and G.N. Cherr. 1996. Sperm motility initiation factor (SMIF) is a minor component of the Pacific herring egg chorion.(In press).

Morisawa, M. 1994. Cell signaling mechanisms for sperm motility. Zool. Sci. 11: 647-662.

Oda, S., Y. Igarashi, H. Ohtake, K. Sakai, N. Shimizu, and M. Morisawa. 1995. Sperm-activating proteins from unfertilized eggs of the Pacific herring, *Clupea pallasi*. Develop. Growth Differ. 37:257-261.

Pillai, M. C., T. S. Shields, R. Yanagimachi, and G. N. Cherr. 1993. Isolation and partial characterization of the sperm motility initiation factor from eggs of the Pacific herring, *Clupea pallasi*. J. Exp. Zool. 265:336-342.

Stacey, N.E., and A.S. Hourston. 1982. Spawning and feeding behavior of captive Pacific herring, *Clupea pallasi*. Can. J. Fish. Aquat. Sci. 39:489-498.

Yanagimachi, R. 1953. Effect of environmental salt concentrations on fertilizability of herring gametes. Jour. Fac. Sci. Hokkaido Univ. Ser. VI (Zool):481-486.

Yanagimachi, R., and Y. Kanoh. 1953. Manner of sperm entry in herring egg, with special reference to the role of calcium ions in fertilization. Jour. Fac. Sci. Hokkaido Univ. Ser. IV (Zool) 11:487-494.

Yanagimachi, R. 1957. Some properties of the sperm-activating factor in the micropyle area of the herring egg. Annotationes Zoologicae Japonenses 30:114-119.

Yanagimachi, R., G. N. Cherr, M. C. Pillai, and J. D. Baldwin. 1992. Factors controlling sperm entry into the micropyles of salmonid and herring eggs. Develop. Growth & Differ. 34:447-461.

STUDY OF MILT IN PUYE *GALAXIAS MACULATUS* (Jenyns, 1848)(SALMONIFORMES: GALAXIDAE)

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ABSTRACT

The sperm density, motility and fertility of milt in "puye" or "angula" (*Galaxias maculatus*, landlocked form) were studied. For this, we used adult specimen taken from Southern Chile's water and kept in captivity for aproximately two years. Once they were mature, twenty specimens (7.33 $\pm$ 0.61cm average total longitude and 1.37 $\pm$ 0.27g average total weight) were anaesthetized and their milt was extracted by stripping. A visual test, sperm recount, motility and fertility evaluation was done to it. The results showed that the puye's milt is not abundant, it is whitish, and has a great viscocity. An average of 61.68 x 10^9 sperm/ml and a motility graded 1, on a 0 to 5 scale (elaborated by Sanchez-Rodríguez & Billard, 1977) in a period of up to 24 hours after it got in contact with freshwater. The fertility average obtained was 92.56 $\pm$ 9.25%. The correlation between fertilization rate with sperm density was 0.177.

Key words: fish, reproduction, milt, sperm, motility, fertility.

INTRODUCTION

The *Galaxias maculatus*, commonly known as "puye or angula", is a small native fish whose crystalline juvenile state (whitebait) is intensely exploited in order to satisfy the national and international market's demand. This has put the species in danger of being extinct and its natural population has collapsed. These recordings show the need of preserving this species in our waters. An alternative is trying to cultivate them for commercial and restocking purposes. To do this, it is fundamental to know and manage diverse stages of this species' life cycle, feeding habits, cultivation conditions, technostructures, and handle in an adecquate way the reproductive processes like spawn, fecundation, and artifitial incubation.

Some aspects of this species have been studied in Chile. Amongst these we find: the life cycle, karyology and migrations (Campos 1970, 1972, 1973), the ovary microstructure and seasonal gonadal activity (Peredo and Sobarzo 1993, 1994), its adaptation to salinity (Vega et al., 1993) and the artificial feeding of larvae (Dantagnan et al., 1995). There are not records on the biology of the sperm of this specie (and of other fish with commercial value). The only research papers on this topic were made by Pérez (1978) and Almendras (1993) who studied the rainbow trout's milt and by Valdebenito et al (1995) who studied the quantitative and cualitative aspects of puye's milt without evaluating its fertility. For this reason, this research's aim is to determine the fertility of *G. maculacus* sperm diluted in freshwater.

MATERIALS AND METHODS

On September 1993, 500 specimens of wild *G. maculatus* adults were captured in the Cautín river near Temuco city, IX region, Chile. The conditioning was done in 100 l capacity, circular, open flow tanks and fed with trout comercial pellets which had to have a lipid adaptation done to them. Once they reached their second sexual maturity (September 1994) a sample of twenty male adults of a total longitude average of 7.33 $\pm$ 0.61cm and 1.37 $\pm$ 0.27g average weight, was taken. They were selected because of their abundant milt produccion. They were then anaesthetized with MS-222 and the maximum milt was extractes by stripping.

Different amounts of the sample were used to determine the sperm density (number of spermatozoa per ml, determined by a recount in Neubauer's camera), the sperm motility in freshwater (according to Sanchez-Rodriguez and Billard, 1977 in scale of 0 to 5)(Table 1) and fertility (percentage of embrions observed after 5 hours of incubation). This last parameter was evaluated in a pool of eggs extracted by stripping 18 adult females (total longitude of 7.99 $\pm$ 0.31cm and 2.22 $\pm$ 0.20g of total weight) taken from the same tank as the males. Each male's milt (aprox. 0.01 ml) was diluted in a petri dish containing 5 ml of freshwater, after that, a sample of aproximately 500 eggs taken from the same pool of eggs, was added to the petri dish. To facilitate the fertilization process, the mixture was stirred for aproximately 30 seconds, then, it was left to settle. After 5hs of incubation the percentage of fecundation was determined under a a stereomicroscope taking a sample of 50 embryos aproximately. The embryos which had 2 or more cells were considered fertilized . The incubation temperature was 12 $\pm$ 1°C.

RESULTS

G. Maculatus milt was whitish and thick, frequently the small volume extracted adhered to its skin, making its extraction and handling more difficult. For this reason, milt was diluted in river water immediately after its extraction.

The quantitative analysis showed an average sperm density of 61.68 $\pm$ 10.3 x 10^9 sperm/ml (maximum 88.8 x 10^9 and a minimum of 50.3 x 10^9 sperm/ml). Motility observed in all the individuals was low and only a small vibration which, according to the Sanchez-Rodriguez and Billard's scale corresponded to grade 1. This motility was prolonged for more than a day at an temperature of 10° C. Although it had a low motility, the spermatozoa was fertile, having an average fecundation percentage of 92.56 $\pm$ 9.25%, evaluating aproximately five hours after the fertilization. The sperm density and fertilization percentage ratio was determined as r = 0.177 (Fig 1).

DISCUSSION

The low flagellar activity observed in all the specimens' spermatozoa studied, is a characteristic which was already observed by Valdebenito et al., (1995). He attributes this to an unknown reproductive strategy of the species or to a condition produced by the stress of captivity, he tried to trigger off the flagellar activity diluting the semen in various solutions, reaching the highest activity rates in a carmabide solution. The low flagellar activity is a characteristic observed in other fish. Ginzburg (1972) reported that the Pacific Herring spermatozoa has a low motility, its beats are aproximately equal to the width of the spermatozoa's head., an energetic movement starts when it is near the egg. Strüssmann et al., (1994) showed that sperm of "pejerrey" (*Odontesthes bonariensis*) remain only feeble vibrations after dilution with ovarias fluid and complete lack of movement has been reported in the fresh milt of *Alosa volgensis*, brook trout,

lake trout and sturgeons (Ginzburg, 1972). He does not report if it is a normal or a pathological characteristic in that species.

The sperm density of 61.68 x 10^9 sperm/ml determined in this research is similar to the one given by Valdebenito et al., (1995). Both results are higher than those given for the rainbow trout (Pérez, 1978), coho salmon, (Bouck & Jacobson, 1976) and Atlantic salmon (Aas et al., 1991), also being a bit higher than the value determined by Grinzburg (1972) for *Perca fluviatilis*, a species that presents a sperm desity of 52 x 10^9 sperm/ml. The high sperm density of the puye may be a biological adaptation to the water currents in which the species lives, Ginzburg (1972) said that species living in strong current waters need a greater number of spermatozoa to fertilize than the species in still water habitats.

Valdebenito et al., (1995), did not evaluate milt's fertility due to the low milt volume produced by each male, but he reported to have used similar milt to the one studied in other experiments, and it was fertile. This observation was proved, in this research, when the good fertility of puye's milt was established, registrating an average fertility percentage of 95.56 $\pm$ 6.46%, a superior value than the one observed by Billard (1990) in trout's spermatozoa. It reached 80% of fertilization when it was diluted in a saline solution and upto 60% when it was diluted in freshwater, higher than the 89.9% of fertility observed by Suquet et al., (1995) en Turbot (*Scophthalmus maximus).*

The high fertility observed in puye's sperm may be helped by the high sperm density used in this investigation. 0.01ml of milt for aproximately 500 eggs was used, this means a total of 1 x 10^6 sperm/egg. This sperm density is quite higher than the one recommended by Billard (1990) for the artificial fertilization of salmonids. He says that the necessary number of spermatozoa needed to fertilize an egg is between 10.000 and 300.000. Suquet et al., (1995) informed that in Turbot, 6 x 10^3 sperm/egg were needed to obtain high fertility percentages. These records were proved by Ginzburg (1972), who points out that a direct relation exists between fertility and sperm density in sturgeons. The high sperm density added to the smaller size of puye's egg (aproximatel 1 mm of diameter after being hidrated) would be the factors that make spermatozoa's fertility better, adding to this, good quality gametes obtained from the females, in which their ovulation time can be detected adequately due to their captivity.

In most salmonides, the spermatozoa's flagellar activity only reaches aproximately 30 sec. when it is diluted in river water (Billsard & Cosson 1988, 1989; Billard 1990 and Perchec et al., 1993), instead, in puye, it is possible to observe a small beat upto 24 hs after it has been diluted in water, after this time the spermatozoa still is viable (Valdebenito, 1996 unpublished). This characteristic is similar to the observations done by Galkina (1957) (in Ginsburg, 1972) who points out that in the *Clupea harengus* the spermatozoa lives 4-5 days in sea water and its fertility reaches 62% after 48 hs of being diluted (Yanagimachi, 1957 in Ginzburg, 1972). The prolonged "flagellar activity" of *G. maculatus* is contrary to Ginzburg's reports (1972) who states that the sperm motility is greater in salty water than in landlocked waters, which could explain why the populatios of *G. maculatus* in landlocked waters still mantain their own milt characteristics that are proper of diadromous populations.

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REFERENCES

Aas, G.H., T. Refstie & D. Gjerde. 1991. Evaluation of milt quality of Atlantic salmon. Aquaculture, 95: 125-132.

Almendras, F.E. 1993. Ensayo de un sistema de criopreservación de semen de salmonídeosaplicado a la trucha arco iris (*Oncorhynchus mykiss*). Tesis para optar al Grado de Licenciado en Medicina Veterinaria. U. Austral de Chile. 46 .

Billard, R. 1990. Artificial insemination in fish. In Marshall's Physiology of reproduction. G. E. Lamming (Ed), 2: 870-888.

Billard, R. & M.P. Cosson. 1988. Sperm motility in rainbow trout, *Parasalmo mykiss*; effect of pH and temperature. Les colloques de l'INRA, 44: 161-167.

Billard, R. & M.P. Cosson. 1989. Measurement of sperm motility in trout and carp. In: Aquaculture, a biotechnology in progress, N. De Pauw, E. Jaspers, H. Ackefors & N. Wilkins (Eds). European Aquaculture Society, Bredene, Belgium : 449-503.

Bouck, G.R. & J. Jacobson. 1976. Estimation of salmonid sperm concentration by microhematocrit technique. Transactions of the American Fisheries Society, 105: 534-535.

Campos, H. 1970. *Galaxias maculatus* (Jenyns) en Chile, con especial referencia a sureproducción. Boletin del Museo Nacional de Historia Natural, Santiago-Chile. 31: 5-20.

Campos, H. 1972. Karyology of three galaxiid fishes *Galaxias maculatus*, *G. platei* and *Brachigalaxias bullocki*. Copeia, 2: 368-371.

Campos, H. 1973. Migration of *Galaxias maculatus* (Jenyns) (Galaxidae: Pisces) in Valdivia estuary, Chile. Hydrobiología, 43(3-4): 301- 312.

Dantagnan, H.P., A. Bórquez, J. Bariles, I. Valdebenito & R. Vega. 1995. Effects of different diets on the survival and growth of puye (*Galaxias maculatus*). Larvi '95-Fish & Shellfish Larvicultures Symposium. Gent, Belgium. P. Lavens, E. Jaspers and I. Roelands (Eds). European Aquaculture Society, Special Publication, (24): 435-437.

Ginzburg, A.S. 1972.Fertilization in fishes and the problem of polyspermy. T.A. Detlaf (Ed).Keter Press. Jerusalem. 365.

Perchec, G., J. Cosson, F. André & R. Billard. 1993. La motilité des spermatozoïdes de truite (*Oncorhynchus mykiss*) et de carpe (*Cyprinus carpio*). Journal of Applied Ichthyology, 9: 129-149.

Peredo, S. & C. Sobarzo. 1993. Microestructura del ovario y ovogénesis en *Galaxias maculatus* (Jenyns, 1842)(Teleostei:Galaxiidae). Biología Pesquera, 22: 23-32.

Peredo, S. & C. Sobarzo. 1994. Actividad gonádica estacional de *Galaxias maculatus* (Jenyns, 1842) en el Río Cautín. IX Región, Chile. Biología Pesquera, 65: 65-70.

Pérez, W.A., 1978. Determinación de las variables seminales fundamentales en la trucha arco iris (*Salmo gairdneri* Gibbons, 1855) en la Piscicultura de Pullinque, Chile. Tesis de Grado para optar al Título de Médico Veterinario. U. Austral de Chile. Valdivia. 29.

Sanchez-Rodriguez, M. & R. Billard. 1977. Conservation de la motilitè et du pouvoir fècondant du sperme de truite arc en ciel maintenu a des tempèratures voisines de 0° C. Bulletin Francais de Pisciculture, (265): 143-152.

Strüssmann, C, Renard Ph, Ling H. & F. Takashima. 1994. Motility of Pejerrey *Odontesthes bonariensis* spermatozoa. Fisheries Science. 60(1): 9-13.

Suquet M, Billard R, Cosson J, Normant Y. & C. Fauvel. 1995. Aritficial insemination in turbot (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of gamete contact. Aquaculture, 133: 83-90

Valdebenito I, Bariles J, Vega R, Dantagnan P, Bórquez A. y E. Carreño. 1995. Análisis cualitativo y cuantitativo del semen de puye *Galaxias maculatus* (Jenyns, 1842)(SALMONIFORMES:GALAXIIDAE).Biologia Pesquera 24:17-21.

Vega, R., A. Pizarro, D. Figueroa, J. Bariles, A. Mardones, S. Peredo, G. Lara, I. Valdebenito & F. Figueroa. 1993. Tolerancia a la salinidad de una población lacustre de puyes *Galaxias maculatus*. Fac. Cs. del Mar. U. Católica del Norte. Coquimbo, Chile. Serie Ocasional 2: 231-238.

Table 1. Index for sperm motility (Adaptade from Sanchez-Rodríguez & Billard, 1977)

Index	Motility characteristics
5	Most spermatozoa display rapid movement; impossible to track the course of any sperm.
4	Most spermatozoa move rapidly while some move slowly.
3	Three classes of spermatozoa can be found; spermatozoa moving rapidly, slowly or vibrating, and those immotile.
2	Most spermatozoa are vibrating or immotile while some present forward movement.
1	Most spermatozoa are immotile and some present lateral vibration
0	All spermatozoa are immotile

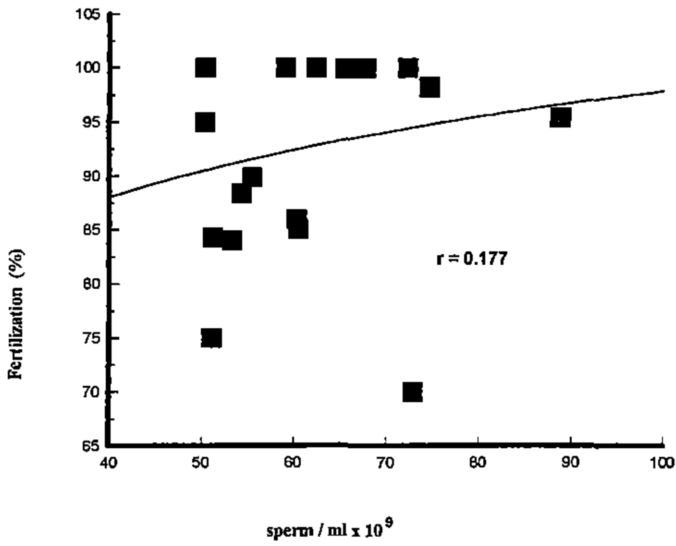

Fig. 1. Relationship between sperm concentration and fertilization (%) in *G. maculatus*.

IN VITRO CORTISOL METABOLISM BY EMBRYONIC TISSUES OF ARCTIC CHARR AND RAINBOW TROUT

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Introduction:

Fish eggs contain several hormones of maternal origin, including thyroid (see reviews by Leatherland, 1994; Lam, 1994) and steroid hormones such as cortisol (Feist et al., 1990; de Jesus and Hirano, 1992; Hwang et al., 1992; Khan et al., 1995). Cortisol is the major interrenal steroid hormone of most teleosts and has been implicated in several physiological processes, including osmoregulation, stress response, and intermediary metabolism. However, little is known about any possible roles of this hormone in the early embryonic development of fish. In mammals, cortisol appears to be needed for differentiation of neuronal and somatic tissues during early ontogeny (Sapolsky and Meaney, 1986; Ballard, 1989; Muglia et al., 1995), but it is neurotoxic at high concentrations (Bohn, 1984; DeKloet et al., 1988; Sapolsky and Meaney, 1986). Therefore, at least in mammals, the level of cortisol to which the fetus is exposed during early development is crucial for normal organogenesis and development (Langlois et al., 1995).

Developing fish embryos, which are dependent on yolk for their nutritional requirements, are probably exposed to the steroid hormones (including cortisol) that are present in the yolk. Whereas in mammals there is an intricate control mechanism involving differential expression of various cortisol-regulating steroidogenic enzymes (Langlois et al., 1995; Nagaya et al., 1995), there is no such information available regarding the the ability of fish embryos to metabolize endogenous cortisol. The purpose of the present study is to determine whether the enzymes involved in cortisol metabolism are expressed in the early developmental stages of embryos of two salmonid fish species.

Although there are no reports on cortisol metabolism in fish embryos, some studies have described the profiles of yolk cortisol during development in various teleosts including Japanese flounder (*Paralichthys olivaceus*) (de Jesus et al., 1991), chum salmon, (*Oncorhynchus keta*) (de Jesus and Hirano, 1992), tilapia (*Oreochromis mossambicus*) (Hwang et al., 1992), rainbow trout (*Oncorhynchus mykiss*) (Yeoh, 1993) and Asian seabass (*Lates calcarifer*) (Sampath-Kumar et al., 1995). There is a decline in the cortisol levels concomitant with the absorption of yolk indicating that the hormone is being eliminated by the embryo as the yolk is absorbed.

The presence of cortisol in yolk could potentially affect the development of the hypothalamus-pituitary gland-interrenal tissue (HPI) axis. The HPI axis in rainbow trout is reported to become responsive to stressor challenge two weeks after hatching and one week before the onset of exogenous feeding (Barry et al., 1995a). Pottinger & Mosuwe (1994) postulated that the appearance of a functional HPI axis is developmentally delayed to avoid stress-induced disruption of other physiological processes during key ontogenetic events. This hyporesponsiveness to stress may be homologous to a similar 2-week postnatal period in rodents, the function of which may be to maintain low, constant corticosteroid levels during a critical development period when these steroids could exert a permanent deleterious affect on neuronal organization (Barry et al., 1995).

We have recently shown that Arctic charr (*Salvelinus alpinus*) embryos are able to metabolize various steroid hormones such as progesterone (P), 17α-hydroxyprogesterone (17αOHP), androstenedione (A_4), testosterone (T), 17β-estradiol (E_2) and estrone (E_1), *in vitro* (Khan et al., 1995; M.N. Khan, R. Renaud and J.F. Leatherland, unpublished). These studies reveal that several key steroidogenic enzymes are expressed early in ontogeny and that steroid hormones are rapidly metabolized. In the present report we present the first evidence of cortisol metabolism by developing embryos of two salmonid species, Arctic charr and rainbow trout.

Materials and methods:

[^{3}H]cortisol incubation

Yolk-sac embryos of Arctic charr [41 days post fertilization (dpf)] and rainbow trout (53 dpf) were killed by decapitation and the yolksac was removed. The embryos (three per well) were placed in individual tissue culture wells (24-well plate, 3.5 ml well capacity, M/S Flow Laboratories Inc., VI, USA). Each well contained 1 ml of modified Medium 199 (M-3274 M/S Sigma Chemical Company) with Hank's salts, glutamine, sodium bicarbonate, bovine albumin, antibiotics and glucose, without phenol red, pH 7.2 (Venkatesh et al., 1992). The radiolabelled substrate [^{3}H]cortisol made in the same medium was added to each well (0.56 nM). Duplicate control wells with no tissue and three replicates (wells) containing fish embryos were used for each incubation time and for each species separately. The plates were gently agitated on a shaker during incubation which lasted for 20 min and 24 h. At the completion of each incubation period the embryos from each well were removed and the incubation media was stored at -20°C until analysed for steroid hormones.

Extraction of steroid hormones

The extraction procedure for HPLC was based on the methods described by Payne et al. (1989), Venkatesh et al. (1989), Kime et al. (1991) and Scott and Canario (1992) with some modifications. The incubate along with embryos was applied to a Sep-Pak C_{18} cartridge which had been primed by washing with 5 ml of methanol and 5 ml of distilled water. The incubate was passed through the Sep-Pak column by syringe and the column was again washed with 5 ml of water and 5 ml of hexane. Subsequently, the free steroid hormones were washed from the column using 5 ml of diethyl ether and the conjugated steroids were eluted with 5 ml of 100 % methanol. The ether and methanol extracts were dried separately under nitrogen at 45°C. The free fraction having radiolabelled metabolites was dissolved in 200 μl of acetonitrile containing a mixture of 21 unlabelled steroid hormones (as internal standards); 20 μl of this mixture was injected onto the HPLC column. The dried conjugated steroid fraction was processed for acid solvolysis and enzyme hydrolysis to obtain the free steroids for HPLC (see below).

Acid solvolysis of sulphates

The dried methanol fraction containing the conjugated steroids was incubated overnight at 45°C with 5 ml of trifluoroacetic acid/ethylacetate (TFA/EA (1/100; v/v) to convert sulphated forms of steroids into free hormones. The solvent was then evaporated and the residue was dissolved in 5 ml of water. The free (formerly sulphated) and glucuronide conjugated steroids were concentrated and separated as described above using Sep-Pak cartridge. The sulphated steroids now present as free steroids, were extracted with 5 ml of diethyl ether. The glucuronide conjugates were extracted with 5 ml of 100% methanol as above and subjected to enzyme hydrolysis.

Enzyme hydrolysis of glucuronides

The dried methanol fraction from the second extraction was reconstituted with 1.0 ml of 0.5 M sodium acetate buffer (pH 5.0) and the glucuronides were hydrolysed at 37°C using 20μl of β-glucuronidase (snail juice, containing 2000 I.U. of enzyme activity). The free steroids resulting from enzymetic hydrolysis were taken up in 5 ml of water and extracted from a Sep-Pak column with 5 ml ether (see above).

HPLC Analysis

The apparatus consisted of a Waters Associates (Milford, MA, USA) Liquid Chromatography System comprising two 510 pumps, a WISP 710B autoinjector and a solvent programmer (Waters Baseline 810 controller Software), coupled with a dual channel model 441 UV detector. The NOVA.Pak C18 (8 mm X 100 mm, 4 μm) column was also purchased from Waters Associates. For optimum separation of steroids Waters Baseline 810 controller Software was used to create a binary solvent gradient of acetonitrile-water. The multistep gradient mobile phase consisted of 29.5, 35, 39, and 75% acetonitrile in water at time 0, 10, 12.5, and 32 min, respectively, in the run. In order to obtain better yield of metabolites off the HPLC column, the incubation replicates were pooled together after extraction, in acetonitrile containing 21 steroid standards and injected into column as described above. Fractions were collected from the HPLC column at 0.5 min intervals for a total of 40 min. To each fraction was added 4 ml of scintillation fluid and the radioactivity was counted on scintillation spectrometer. Retention times for each steroid hormone was determined from the elution time of the internal standards and the absorbance was monitored at 254 nm and 280 nm. The counts (CPM) in each fraction and absorbance of 21 internal standards was plotted against retention time.

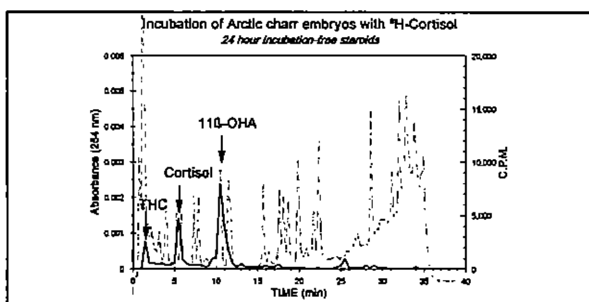
Results:

Both Arctic charr (41 dpf) and rainbow trout (53 dpf) embryos converted [^{3}H]cortisol to 11β-hydroxyandrostenedione (11β-OHA). Another metabolite, more polar than cortisol, probably tetrahydrocortisol or tetrahydrocortisone, was also found eluting immediately after the solvent front. The elution profiles of cortisol and its metabolite as separated by HPLC for both species are shown in Table 1 and Figure 1-2. The metabolite yields were maximal after 20 min of incubation and remained in the same range throughout the incubation time, indicating that 11β-OHA is the end product of cortisol metabolism in salmonid embryos. In addition to 11β-OHA, a small fraction of cortisol was also converted to its sulphated and glucuronated metabolites.

Table 1. *In vitro* metabolism of [^{3}H] cortisol (0.56 nmol/well) by yolk sac embryos of Arctic charr (41 dpf) and rainbow trout (53 dpf) after 20 min and 24 h of incubation at 10°C. The radioactivity (CPM) measured under the respective peak area and expressed as % of total counts.

		Arctic charr	**Rainbow trout**
Metabolites	**Time**	**41 dpf**	**53 dpf**
Cortisol (substrate)	20 min	14.5	31.0
	24 h	21.62	26.2
11β-OHA*	20 min	52.4	44.5
	24 h	54.9	46.8
Tetrahydrocortisol (?)	20 min	18.5	14.5
	24 h	16.1	13.6
Sulphates	20 min	11.4	7.0
	24 h	5.7	8.9
Glucuronides	20 min	3.0	3.1
	24 h	1.6	4.5

* 11β-hydroxyandrostenedione



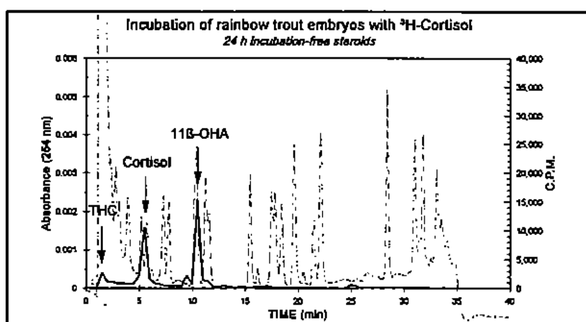


Figure 1 (upper) 2 lower. HPLC profiles of radiolabelled steroid hormones produced by Arctic charr (Fig. 1) and rainbow trout (Fig. 2) embryos after incubation for 24 h with [^{3}H]cortisol. THC = tetrahydrocortisol or tetrahydrocortison (?); 11β-OH-A = 11β-hydroxyandrostenedione.

Discussion:

Arctic charr and rainbow trout embryos appear to convert [^{3}H]cortisol almost exclusively to 11β-OHA, with the exception of a smaller peak of unknown metabolite probably a tetrahydro derivative of cortisol or cortisone. This finding is consistant with the report of Kime (1978) who demonstrated that cortisol, incubated *in vitro* with liver slices of rainbow trout, pike (*Esox lucius*) and perch (*Perca fluviatilis*), was converted into cortisone, 11β-OHA and 11-oxoandrostenedione.

Cortisol is known to be secreted as part of the physiological stress response in fish, and may have inhibitory effects on steroidogenesis (Pankhurst and Dedual, 1994). Similarly, in mammals, glucocorticoids are reported to be neurotoxic at high concentrations during fetal development (Sapolsky and Meaney, 1986). Therefore, it seems plausible to speculate that the same hormone may also elicit a deleterious stress-response in the sensitive early embryonic stages in fish. The fact that exogenous cortisol was converted to 11β-OHA further indicates that fish embryos may

be able to inactivate cortisol and thus avoid these effects. In this context, it is interesting to note that appearance of a functional HPI axis in salmonids has been reported to be delayed possibly an adaptation to avoid the stress-induced disruption of other physiological processes during the key ontogenetic events (Pottinger and Mosuwe, 1994). A parallel situation has been reported in mammals in which a nadir in responsiveness to adrenocorticotrophic hormone (ACTH) acts to lower the exposure of the developing fetus to bioactive glucocorticoids (Langlois et al., 1995). In our experiments, the (yolk sac) embryos of rainbow trout and Arctic charr were incubated with cortisol at a time in development when the HPI axis is thought not to be functional (Barry et al., 1995).

We have recently found that in Arctic charr embryos there are various steroidogenic enzymes expressed very early in the development (Khan et al., 1995) even before the reported maturity of the HPI axis in similar salmonids (Saga et al., 1993; Barry et al., 1995). One of these enzymes, 17, 20-desmolase (lyase) [also called mitochondrial cytochrome P450 enzyme (side chain cleavage, $P450_{scc}$)], converts 17αOHP to A_4, and can also cleave the cortisol molecule to form A_4. The A_4 produced as an intermediate metabolite can then be converted to 11β-OHA by 11β-hydroxylase [also called mitochondrial cytochrome P450 enzyme (11hydroxylase, $P450_{c11}$)]. These two enzymes are possibly being expressed *de novo* in response to [^{3}H]cortisol challenge before the maturation of the HPI axis; therefore, the catabolism of the cortisol may reflect the need of the embryo to excrete the hormone to protect itself from the adverse effects of this biologically potent steroid hormone. This finding is in agreement with our recent work which has established that Arctic charr embryos can inactivate several bioactive steroid hormones such as P_4, 17α-OHP, T, E_2 and E_1 (Khan et al., 1995; M.N. Khan, R. Renaud, and J.F. Leatherland, unpublished). The catabolism of cortisol to an inactive metabolite, 11β-OHA, therefore may represent another example of this inactivation process. This hypothesis is further supported by the finding in mammals that the expression of the same enzymes, $P450_{scc}$ and 11β-hydroxylase regulates the cortisol levels in neonatal rats (Nagaya et al., 1995). Similarly, the expression of two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) which convert bioactive cortisol to the less active cortisone in developing ovine fetal liver and kidney, is thought to protect the fetus from elevated levels of glucucorticoids (Langlois et al. 1995). Another enzyme, 20β-reductase, has also been shown to be expressed in the chorioallantoic membrane of chick embryos (McNatt et al., 1992); this enzyme metabolizes cortisol to produce 20β-dihydrocortisol, and both of these hormones are angiostatic (i.e., they inhibit neovascularization of the chorioallantoic membrane); the expression of this enzyme perhaps controls the development of the vascular capillary network in chick embryo. The expression of $P450_{scc}$ and 11β-hydroxylase in fish embryos that are exposed to exogenous (maternal) cortisol, and the resulting conversion of cortisol to 11β-OHA may represent a parallel of the phenomena seen in mammalian and avian models.

The other minor, as yet unidentified, metabolite in the present experiment may be one of tetrahydro derivatives of cortisol or cortisone. McKerns (1969) reported that in human beings the major excreted metabolite of cortisol is tetrahydrocortisol or tetrahydrocortisone. Similarly Pottinger et al. (1992) found that the predominant steroid metabolite in the bile of stressed fish is tetrahydrocortisone. The four hydroxyl groups would make this metabolite more polar than cortisol, and it would therefore elute from the HPLC column before the cortisol/cortisone. However, the definite identity of this metabolite could not be ascertained due to lack of a proper standard.

Conjugation as a form of inactivation does not seem to be the preferred mode of cortisol

metabolism in either Arctic charr or rainbow trout embryos. It is possible that unlike other steroids, for example T, which is suitable substrate for conjugation without prior transformation (Förlin and Haux, 1985; Andersson et al., 1985), cortisol undergoes extensive metabolism without conjugation (Truscott, 1979; Pottinger et al., 1992). Therefore, in embryos of the two salmonid species examined here, the main route of detoxification of cortisol appears to be *via* the formation of 11β-OHA and possibly tetrahydrocortisol/tetrahydrocortisone, rather than by conjugation.

REFERENCES:

Andersson, T., Pesonen, P., and Johansson, C. (1985). Differential induction of cytochrome P-450-dependant monooxygenase, epoxide hydrolase, glutathione transferase and UDP glucuronosyl transferase activities in the liver of rainbow trout by β-naphthoflavone or clophen A50. Biochem. Pharmacol. 34, 3309-3314.

Ballard, P.L. (1989). Hormonal regulation of pulmonary surfactant. Endocrine Rev. 10, 165-181.

Barry, T.P., Malison, J.A., Held, J.A., and Parish, J.J. (1995). Ontogeny of the cortisol stress response in larval rainbow trout. Gen. Comp. Endocrinol. 97, 57-65.

Bohn, M.C., (1984). Glucocorticoid induced teratoloies of the nervous system. In: Neurobehavioral Teratology, J. Yanai (Ed.), Elsevier Science Publishers BV, Amsterdam, pp. 365-388

DeKloet, E.R., Rosenfeld, P., Van, Eekelen, J. A.M., Sutanto, W., and Levine, S. (1988). Biochemical basis of functional neuroteratology. Progr. Brain Res. 73, 101-120.

de Jesus, E.G.T., Hirano, T., and Inui, Y. (1991). Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. Gen. Comp. Endocrinol. 82, 369-376.

de Jesus, E.G.T., and Hirano, T. (1992). Changes in whole body concentrations of cortisol, thyroid hormones, and sex steroids during early development of the chum salmon, *Oncorhynchus keta*. Gen. Comp. Endocrinol. 85, 55-61.

Feist, G., Schreck, C.B., Fitzpatrick, M.S., and Redding, J.M. (1990). Sex steroid profiles of coho salmon (*Oncorhynchus kisutch*) during early development and sexual differentiation. Gen. Comp. Endocrinol. 80, 229-313.

Förlin, L., and Haux, C. (1985). Increased excretion in the bile of 17β-[^{3}H]estradiol-derived radioactivity in rainbow trout treated with β-naphthoflavone. Aqu. Toxicol. 6, 197-208.

Hwang, P.-P., Wu, S.-M., Lin, J.-H., and Wu, L.-S. (1992). Cortisol content of eggs and larvae of teleosts. Gen. Comp. Endocrinol. 86, 189-196.

Khan, M.N., Renaud, R., and Leatherland, J.F. (1995). Correlation between plasma and egg steroid hormone content of Arctic charr. In: Proc. Vth Int. Symp. Rep. Physiol. Fish, Austin, Texas, F.W. Goetz and P. Thomas (Eds.), p. 366.

Kime, D.E. (1978). The hepatic catabolism of cortisol in teleost fish-Adrenal origin of 11-

oxotestosterone precursors. Gen. Comp. Endocrinol. 35, 322-328.

Kime, D.E., Venkatesh, B., and Tan, C.H. (1991). 5α-pregnane-3β,7α,17,20α- and -20β-tetrols as metabolites of progesterone and 17-hydroxyprogesterone in carp (*Cyprinus carpio*) ovarian incubations. Gen. Comp. Endocrinol. 84, 401-404.

Lam, T.J. (1994). Hormones and egg/larval quality in fish. J. World Aqualt. Soc. 25, 2-12.

Langlois, D.A., Matthews, S.G., Yu, M., and Yang, K. (1995). Differential expression of 11β-hydroxysteroid dehydrogenase 1 and 2 in the developing ovine fetal liver and kidney. J. Endocrinol. 147, 405-411.

Leatherland, J.F. (1994). Reflections on thyroidology of fishes: from molecules to humankind. Guelph Ichthyol. Rev. 2, 1-67

McKerns, K.W. (1969). Catabolism and inactivation of steroid hormones by the liver and kidney. In: Steroid Hormones and Metabolism. Appleton-Century-Crofts, New York, pp. 93-103.

McNatt, L.G., Lane, D., and Clark, A.F. (1992). Angiostatic activity and metabolism of cortisol in the chorioallantoic membrane (CAM) of the chick embryo. J. Steroid Biochem. Molec. Biol. 42, 687-693.

Muglia, L., Jacobson, L., Dikkes, P. and Majzoub, J.A. (1995). Corticotropin-releasing hormone deficiency reveals major fetal but not adult glucocorticoid need. Nature 373, 427-431.

Nagaya M., Arai, M., and Widmaier, E.P. (1995). Ontogeny of immunoreactive and bioactive microsomal steroidogenic enzymes during adrenocortical development in rats. Mol. Cell. Endocrinol. 114, 27-34.

Pankhurst, N.W., and Dedual, M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in natural population of rainbow trout *Oncorhynchus mykiss*. J. Fish Biol. 45, 1013-1025.

Payne, D.W., Holtzclaw, W.D., and Adashi, E.Y., (1989). A convenient unified scheme for the differential extraction of conjugated and unconjugated serum C_{19} steroids on Sep-Pak C_{18}-cartridges. J. Steroid Biochem., 33, 289-295.

Pottinger, T.G., and Mosuwe, E. (1994). The corticosteroidogenic response of brown and rainbow trout alevins and fry to environmental stress during a "critical period". Gen. Comp. Endocrinol. 95, 350-362.

Pottinger, T.G., Moran, T.A., and Cranwell, P.A. (1992). The biliary accumulation of corticosteroids in rainbow trout, *Oncorhynchus mykiss*, during acute and chronic stress. Fish Physiol. Biochem. 10, 55-66.

Saga, T., Oota, Y., Nozaki, M., and Swanson, P. (1993). Salmonid pituitary gonadotrophs. III. Chronological appearance of GTH I and other adenohypophysial hormones in the pituitary of the developing rainbow trout (*Oncorhynchus mykiss irideus*). Gen. Comp. Endocrinol. 92, 233-241.

Sapolsky, R.M., and Meaney, M.J. (1986). Maturation of the adrenal stress response: Neuroendocrine control mechanisms and the stress hyporesponsive period. Brain Res. Rev. 11, 65-76.

Sampath-Kumar, R., Byers, R.E., Munro, A.D., and Lam T.J. (1995). Profile of cortisol during the ontogeny of the Asian seabass, *Lates calcarifer*. Aqualture 132, 349-359.

Scott, A.P., and Canario, A.V.M. (1992). 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate: A major new metabolite of teleost oocyte maturation-inducing steroid. Gen. Comp. Endocrinol. 85, 91-100.

Sumpter, J.P., Carragher, J.F., Pottinger, T.G., and Pickering, A.D. (1987). Interaction of stress and reproduction in trout. In: Reproductive Physiology of Fish. D.R. Idler, L.W. Crim, and J.M. Walsh (Eds.), Memorial Univ. of Newfoundland, St John's, pp. 299-302.

Truscot, B. (1979). Steroid metabolism in fish. Identification of steroid moieties of hydrolyzable conjugates of cortisol in the bile of trout *Salmo gairdneri*. Gen. Comp. Endocrinol. 38, 196-206.

Venkatesh,B., Tan, C.H., and Lam, T.J. (1989). Blood steroid levels in the goldfish: measurement of six ovarian steroids in small volumes of serum by reverse-phase high-performance liquid chromatography and radioimmunoassay. Gen. Comp. Endocrinol. 76, 398-407.

Venkatesh, B., Tan, C.H., Kime, D.E., and Lam, T.J. (1992). Steroid metabolism in teleost gonads: purification and identification of metabolites by high-performance liquid chromatography. Steroids 57, 276-281.

Yeoh, C.-G. (1993). The effects of hormone on development of embryonic and postembryonic salmonids, and hormone metabolism during these stages. (M.Sc. Thesis Oregon State University, USA).

EFFECT OF HCG DOSAGES ON HATCHING SUCCESS IN WHITE BASS

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Background

Research related to the development of commercial aquaculture of *Morone* species has focused increasingly on the culture of striped bass *M. saxatilis* x white bass *M. chrysops* hybrids. Numerous studies has demonstrated that both hybrids, the female striped bass x male white bass (palmetto bass) and the female white bass x male striped bass (sunshine bass), are faster growing (at least during the first 2 years of life), more robust, and more resistant to disease and environmental extremes than purebred striped bass (Kerby, 1986).

Recent declines in the striped bass fisheries along the Atlantic coast of the United States, as well as legal and regulatory constraints, have increasingly limited the availability of wild broodstock (especially females) as a source of gametes (Harrell, 1984). In part, the problem of limited availability of striped bass eggs could be greatly mediated by using female white bass crossed with male striped bass to produce reciprocal-cross sunshine bass hybrids. White bass are common in much of North America (Scott and Crossman, 1973; Becker, 1983); their range has been greatly expanded as a result of stocking. However, legal and regulatory constraints may also limit access to wild white bass stocks. To resolve the dilemma of gamete availability, strategies for domesticating broodstocks and associated methods of controlling reproduction need to be developed (Donaldson and Hunter, 1983; Idler et al., 1987).

Two additional benefits of using female white bass as broodstock are that they normally mature at an earlier age (age 2) and are much smaller (i.e., 250-580 g body weight) than female striped bass,

which in the wild typically mature at age 4 or older and weigh 2-7 kg of more (Scott and Crossman, 1973; Bonn et al., 1976). Earlier age at maturity and smaller size are characteristics that should greatly reduce the cost and effort of broodstock rearing and maintenance.

Strategies for domesticating white bass broodstocks and associated methods of controlling reproduction have been developed (Kohler et al., 1994). In the course of this research it became apparent that the traditional hCG dosage (i.e., 1100 IU/kg wet weight female white base), which is 2-3 times higher than that used for striped bass, had not been adequately tested for efficacy. Accordingly, we evaluated hCG dosages ranging from 0-1100 IU/kg in white bass females that had been habituated to captivity and brought into spawning condition through temperature and photoperiod control.

Methods

Adult white bass (300-600 g) were collected from the Illinois River near LaSalle, Illinois, via hook-and-line fishing. Fish were placed in aerated live wells upon capture, moved to an oxygenated, truck-mounted hauling tank, and transported (5-6 h) to several indoor 10,000-L water-recycle systems at Carbondale, Illinois.

Fish were trained to formulated feed initially with moist pellets, which were prepared by mixing a commercial dry trout feed (Purina Trout Chow "broodstock" diet: 40% crude protein, 11% crude fat), raw gizzard shad *Dorosoma cepedianum,* and vitamins (coldwater fish premix). The proportion of dry feed was slowly increased in the diet until fish accepted 100% dry feed, a process that usually took about 2 weeks.

Spawning of white bass occurs between 15-20 °C (Kohler et al., 1994). Water temperatures of all systems were controlled by 746-W (1.0 horsepower) chillers (Frigid Unit) or 1,200-W heating elements placed in each tank, as needed. Temperatures were measured by continuous thermal recorders (Ryan TempMentor model RTM) placed in each system. Rock salt (NaCl) was added to the systems to maintain salinity at about 2%. Dissolved oxygen, temperature, salinity, pH, nitrites, total ammonia, and chlorine were routinely measured, and values obtained were consistently suitable for maintaining good fish health (Stickney and Kohler, 1990).

We attempted to induce spawning with hCG injections at dosages of 0 to 1,100 IU/kg for female fish and 100 IU/kg for male fish. Before injection, the fish were anesthetized with tricaine methanesulfonate (MS-222); 50-100 mg/L) and weighed to the nearest gram. The injections were administered intramuscularly just ventral to the first dorsal fin above the lateral line. Fish were given unique marks (dorsal spine or caudal fin clips) for subsequent identification.

Female white bass were checked for ovulation every 2 h from 16 h post-hCG injection by lightly exerting abdominal pressure to extrude a small amount of eggs. Eggs were staged by procedures similar to those described for striped bass (Kerby, 1986; Rees and Harrell, 1990). In general, ovulation was indicated by the occurrence of clear, free-flowing, uniform-shaped eggs with fully intact inner chorion surfaces. Females that had ovulated were anesthetized with MS-222 (50-100 mg/L), weighed, and dried with a paper towel. We manually stripped eggs (approximately 80% of egg mass) into weighed Teflon cups (15 mL volume). The egg-containing cups were weighed to the nearest 0.1 g, and the number of eggs was estimated by subsampling (Rees and Harrell, 1990). Semen was collected from males by inserting a pasteur pipette in the urogenital opening and applying suction. Semen from two males was placed in the Teflon cups with the eggs of each female, water was added at twice the egg volume, and the contents of the cups were mixed.

After fertilization (2 min), the eggs were placed in a modified Heath tray in labeled, 6 x 6 cm

individual compartments constructed from poly-vinyl chloride plastic and 125 -μm-mesh Nitex screen. Approximately 5,000 eggs were placed in each compartment. A continuous flow of 16-18°C oxygenated water (5 L/min) was circulated through the trays. Trays were covered with black plastic sheeting to prevent damage to eggs and larvae from excessive light. The eggs generally began to hatch 36-48 h postfertilization, and hatching was completed within 24 h. An additional 96-120 h was required for the larvae to absorb their yolk sacs. All live larvae, dead larvae, and unhatched eggs from each spawn were fixed in 10% formalin for several days and then placed in separate, labeled jars containing 95% ethanol. Eggs were counted with a Plexiglas counting chamber and a dissecting microscope at 10 X magnification.

Results and Discussion

hCG dosages considerably less than those traditionally used to induce ovulation in white bass appear to be more efficacious (Table 1). Based on these results we suggest that dosages similar or lower to those used for striped bass (330 IU/kg; Kerby, 1986), be used for white bass.

Table 1. Ovulation time and percent hatch for white bass induced to spawn using various dosages of hCG.

hCG Dosage/Female	Ovulation Time (h)		% hatch	
(IU/kg wet weight)	Mean	Range	Mean	Range
1100	24	16-32	22	0-58
830	31	26-45	36	6-90
280	28	24-34	58	0-90
250	39	37-40	63	36-81
170	25	21-29	61	15-89
150	38	29-47	44	19-64
50	37	34-39	73	66-89
0*	--	--	--	--

* Did not spawn.

In addition to providing guidance for improved spawning performance, these data have positive implications toward eventual regulatory approval of hCG for spawning *Morone* spp.

References

Becker, GC 1983 Fishes of Wisconsin. University of Wisconsin Press, Madison.

Bonn, EW, WM Bailey, JD Bayless, KE Erickson, and RE Stevens, editors. 1976. Guidelines for striped bass culture. American Fisheries Society, Southern Division, Striped Bass Committee, Bethesda, Maryland.

Donaldson, EM, and GA Hunter 1983 Induced final maturation, ovulation, and spermiation in cultured fish. Pages. 351-403 *in* W.S. Hoar, D.J. Randall, and E.M. Donaldson, editors. Fish physiology, volume 9, part B. Academic Press, New York.

Harrell, RM 1984 Tank spawning of first generation striped bass × white bass hybrids. Progressive Fish-Culturist 46:75-78.

Idler, DR, LW Crim, and JM Walsh, editors 1987 Proceedings of the third international symposium on the reproductive physiology of fish. Memorial University of Newfoundland, St. John's.

Kerby, JH 1986 Striped bass and striped bass hybrids. Pages 127-147 *in* R.R. Stickney, editor. Culture of nonsalmonid freshwater fishes. CRC Press, Boca Raton, Florida.

Kohler, CC, RJ Sheehan, C Habicht, JE Malison, and TB Kayes 1994 Habituation to captivity and controlled spawning of white bass. Transactions of the American Fisheries Society 123:964-974.

Rees, RA, and RM Harrell 1990 Artificial spawning and fry production of striped bass and hybrids. Pages 43-72 *in* R.M. Harrell, J.H. Kerby, and R.V. Minton, editors. Culture and propagation of striped bass and its hybrids. American Fisheries Society, Southern Division, Striped Bass Committee, Bethesda, Maryland.

Scott, WB, and EJ Crossman 1973 Freshwater fishes of Canada. Bulletin of the Fisheries Research Board of Canada 184.

Stickney, RR, and CC Kohler 1990 Maintaining fishes for research and teaching. Pages 633-663 *in* C.B. Schreck and P.B. Moyle, editors. Methods for fish biology. American Fisheries Society, Bethesda, Maryland.