Environmental Influences on Reproduction
TEMPERATURE MANIPULATION OF SEX DIFFERENTIATION IN FISH

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Summary

This paper reviews the effects of the thermal environment on the primary sex differentiation of fishes, with special regard to the evidence of thermolabile sex-determination (TSD). TSD is common among Atherinoids, and temperature effects on gonadogenesis in this group range from functional sex change to virtual sterilization. Within this group, exposure to extreme (sub-lethal) temperatures seems to allow sex manipulation even in species with otherwise strong genetic sex determination (GSD). In other taxa, including several economically important species, there are instances in which (phenotypical) gonadal sex appears at least partly modulated by environmental temperature. Practical considerations for the demonstration of TSD and its prospects for the control of fish sex in aquaculture are discussed.

Introduction

Environmental factors define the reproductive strategies and the output of fish reproductive activity by setting the timing and the amount of energy available for reproduction (Lam, 1983). In a number of species, environmental factors also influence the relative contribution of each sex to reproduction by temporally or permanently inducing functional sex-change (environmental sex determination, ESD). The occurrence of the various forms of ESD among fishes (temperature-, behavioral-, salinity-, light-, water quality-, pH-, or nutrition-dependent sex determination) has been dealt with in a number of reviews (Chan and Yeung, 1983; Adkins-Reagan, 1987; Korpelainen, 1990; Shapiro, 1990; Francis, 1992). Thermolabile sex determination (TSD) seems to be the prevalent form of ESD among fishes and other animals in general (Korpelainen, 1990). Although most authors agree that TSD is likely widespread among teleosts (Conover, 1984, 1992; Schultz, 1993; Adkins-Reagan, 1987; Korpelainen, 1990; Francis, 1992), reports of TSD are still scarce (Schultz, 1993). One reason is that demonstration of TSD in fish imposes practical difficulties such as poor survival (Schultz, 1993; see also below). Another reason may be that TSD is generally viewed in an ecological or evolutionary context, with much emphasis being placed in demonstrating its adaptive significance. Only recently have scientists realized the broader implications and potential of TSD for aquaculture and hence studies in this area are gradually being undertaken. This review deals with TSD and other effects of temperature on the gonadogenesis of gonochoristic fishes, particularly in atherinoids where most studies have been conducted. We also discuss the practical use of TSD in aquaculture.

Effects of temperature on sex differentiation of atherinids

Perhaps the most thoroughly studied and best known example of TSD in fishes occurs in one member of the family Atherinidae, the Atlantic silverside, Menidia menidia. Individuals of this species become male or female in response to the temperature regime experienced during a specific period in early life (Conover and Kynard, 1981; Conover and Fleisher, 1986). Presumably, TSD confers an adaptive advantage for M. menidia by the creation of sex-linked size dimorphism. Females are born earlier than males as a result of incubation under the low temperatures prevailing in the beginning of the spawning season and thus have a longer growing season, becoming larger than males at the time of sexual maturation. Size has a relatively greater effect on the fecundity of females than that of males (Conover, 1984, 1992). Interestingly, it was shown that environmental sex-determination is itself under genetic control (Conover and Kynard, 1981; Conover and Heins, 1987a). Also, the degree of environmental versus genetic sex-determination and the magnitude of the response to temperature varied among local populations of M. menidia, reflecting an adaptive compensation to local differences in the thermal environment (Conover, 1992; Conover and Heins, 1987b). The gonadal sex of Northern populations of M. menidia, for instance, was completely unresponsive to temperature (Fig. 1).

TSD has been subsequently demonstrated in three other atherinids, the tidewater silverside, M. pennisulcata (Middaugh and Hemmer, 1987), and two South American silversides from mean latitudes, Odontesthes bonariensis (Strössmann et al., 1995a), a freshwater and estuarine species, and O. argentinensis (Strössmann et al., unpublished data), a marine species. A fifth species, Patagonina hatcheri, an inhabitant of the cold freshwaters of Patagonia, initially appeared unresponsive to temperature and was therefore considered strongly genetically sex determined (Strössmann et al., unpublished data). However, recent studies have succeeded in manipulating the sex of P. hatcheri by use of even lower temperatures (Saitoh and Strössmann, unpublished data). The adaptive significance of TSD for these species, if any, remains unclear (Strössmann et al., 1995a) or conjectural at best (Middaugh and Hemmer,
In all these species, temperature effectively modifies gonadal sex only when applied at a specific ontogenetic stage. This stage has been determined to be around metamorphosis and it culminates with the period of histological sex differentiation of the gonads (Conover and Fleisher, 1986; Strüssmann et al., 1995a, b). All atherinid species examined seem to be gonochorists and intersexes are rare. Thus, temperature treatments before or after the phenocritical stage of sex determination do not alter sex.

Effects of temperature on sex differentiation in other species

Evidence suggesting environmentally-related modifications of phenotypical sex among other fish groups are available from a number of sources. Circumstantial evidence can be drawn from some studies aimed at the detection of sex chromosomes as well as from those attempting the production of monosex fish by mating of sex-reversed parents or by gynogenesis induction. In short, the majority of these studies have produced sex ratios which conformed most of the time to simple genetic sex inheritance systems (XX-XY or ZW-ZZ) but occasionally with a few inexplicable exceptions (Betta splendens, Lowe and Larkin, 1975; Brachydanio rerio, Streisinger et al., 1981; Carassius auratus, Oshiro, 1987; Gnathopogon caudescens, Fujioka, 1993; Carassius carassius grandoculis, Fujioka et al., 1995). Some of these studies even suggested a possible link between water temperature and variable sex ratios. For instance, Winge (1934) and Aida (1936) noted the formation of higher proportions of males in Poecilia reticulata and Oryzias latipes, respectively, during the warmer months. It is unclear whether all these exceptional cases are evidence of thermal (or other form of environmental) sex reversal, but some were subsequently shown to be (see below).

More direct evidence of TSD has since being produced by direct comparison of the sex ratios of broods reared under controlled thermal conditions. Although limited in scope, Table 1 shows that trials with either normal or putative monosex progeny of various species sometimes produced significantly different sex ratios at different temperatures. It must be noted that several of these species have been previously assumed to possess stable genetic sex determining mechanisms. In fact, the mostly biased sex ratios of putative monosex (Table 1) seem to support a strong role for genotypic sex determination (GSD) in these species. Even in such species, however, temperature appears able to override genetic sex determination. It is not known whether all the unsuccessful trials reflect parental genotypes that are unresponsive to temperature, such as in the case of Poeciliopsis lucida (Sullivan and Schultz, 1986; Schultz, 1993), or simply those with different thermal thresholds for TSD. Low temperatures favor the production of females in some species, including all atherinids previously described, whereas in others the reverse is the case (Table 1).

The direction of the response to temperature is markedly constant among atherinids: higher temperatures favor the formation of males whereas lower temperatures favor the formation of females (Conover and Kynard, 1981; Middaugh and Hemner, 1987; Strüssmann et al., 1995a, Fig. 1). Transition from female-producing to male-producing temperatures appears monotonic and gradual (Conover and Heins, 1987a; Fig. 1), in contrast to the steep threshold or sometimes "U-shaped" response of some reptiles (Korpela, 1990). In *Patagonina hatcheri*, however, it appears that threshold temperatures occur at the lower range of viable temperatures. In this species, sex-ratios are remarkably variable from 1:1 between 25 and 17°C but nearly 90% females can be produced at 15°C (Strüssmann et al., unpublished data).

**Fig. 1.** Relation of water temperature during the period of sex determination and sex-ratio in some atherinid species. Means from data of Conover and Heins (1987b; *M. menidia*) and Strüssmann et al. (1995a, unpublished data).
Table 1 Effects of rearing temperature on sex ratios of fishes other than atherinids.

<table>
<thead>
<tr>
<th>Species</th>
<th>Successful trials*</th>
<th>low mean temperatures</th>
<th>high mean temperatures</th>
<th>Effective temp. to produce雌 females &amp; males</th>
<th>Type of brood used*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gasterosteus aculeatus</td>
<td>1/1</td>
<td>55.9 (16-20)</td>
<td>80.2 (19)</td>
<td>low normal bisexual</td>
<td>Lindsey, 1962</td>
<td></td>
</tr>
<tr>
<td>Oreochromis mossambicus</td>
<td>2/2</td>
<td>22.0 (20)</td>
<td>42.4 (26)</td>
<td>low normal bisexual</td>
<td>Mair et al., 1990</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.1 (19)</td>
<td>85.1 (25)</td>
<td>low probably putative all-female brood</td>
<td>Mair et al., 1990</td>
<td></td>
</tr>
<tr>
<td>O. aureus</td>
<td>1/7</td>
<td>4.8 (29)</td>
<td>20.0 (25)</td>
<td>low putative all-female brood</td>
<td>Sullivan and Schultz, 1986</td>
<td></td>
</tr>
<tr>
<td>Poecilopterus lucida</td>
<td>1/2</td>
<td>62.0 (24)</td>
<td>8.0 (30)</td>
<td>high normal bisexual</td>
<td>Oshiro et al., 1988</td>
<td></td>
</tr>
<tr>
<td>Carassius auratus</td>
<td>4/6</td>
<td>80-100 (24)</td>
<td>- (25)</td>
<td>high putative all-female brood</td>
<td>Fujikawa, 1995</td>
<td></td>
</tr>
<tr>
<td>C. carassius granuloculis</td>
<td>2/6</td>
<td>50.0 (19-21)</td>
<td>21.4 (28-31)</td>
<td>high normal bisexual</td>
<td>Yanamoto and Masutani, 1990</td>
<td></td>
</tr>
<tr>
<td>Fundulus olivaceus</td>
<td>2/2</td>
<td>- (20)</td>
<td>10.0 (25)</td>
<td>high normal bisexual</td>
<td>Nomura et al., 1994</td>
<td></td>
</tr>
<tr>
<td>Messemarchus anguillicaudatus</td>
<td>3/3</td>
<td>- (20)</td>
<td>60.0 (25)</td>
<td>high putative all-female brood</td>
<td>Ochiai et al., 1994</td>
<td></td>
</tr>
<tr>
<td>Oryzias latipes</td>
<td>1/1</td>
<td>- (20)</td>
<td>NS (30)</td>
<td>high putative all-female brood</td>
<td>Fujikawa, 1993</td>
<td></td>
</tr>
<tr>
<td>Gonionotoxus carinatus</td>
<td>1/1</td>
<td>- (20)</td>
<td>70.0 (30)</td>
<td>high putative all-female brood</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Trials that produced significantly different sex ratios at variable temperatures over the total number of trials performed. Each trial used progeny from different parents.
+ Results of successful trial(s). Actual temperatures are given within parenthesis. NS: not specified.
+ As concluded by the authors of these studies.
+ Some studies used putative monosex broods produced by gynogenesis or from sex-reversed broodstock.

Temperature-dependent sterilization

Thermally sex-reversed fish generally have normal gonadal histology and are presumed to be functional. However, Strössmann et al. (1995a, unpublished data) noted the occasional occurrence of individuals of O. bonariensis and P. hatcheri whose gonads were poor in or completely devoid of germ cells in groups reared at the high temperatures. Current research has since examined this problem and established that prolonged exposure to temperatures around 29°C causes the disappearance of germ cells in both male and female gonads of O. bonariensis (Strössmann and Saijto, unpublished data; Fig. 2). Since at 29°C only males are formed, sterilization of female-gonads involves previous exposure to lower temperatures during sex-differentiation. Conversely, formation of normal testes in all-male groups requires short-term exposure to high temperatures. Insufficiently high temperatures or short exposures to 29°C seem to cause two types of partial germ cell disappearance: either their number is greatly reduced or they disappear completely from only one of the two lobes of the gonads (Strössmann and Saijto, unpublished data).

Warm temperature-sterilization likely occurs in other fish groups. For instance, Nakamura and Nagahama* recently reported the nearly complete elimination of germ cells in the gonads of Oreochromis niloticus exposed to 36-37°C between 7-50 days after hatching. They also showed that these effects are permanent and resulted in sterile adults.

Thermal manipulation of sex in aquaculture

From what has been described above, i.e. that thermal liability of sex appears widespread and that thermal manipulation of sex may allow both functional sex reversal and production of neutered fish, the possibilities of application of TSD for aquaculture appear extraordinary. In many species, uncontrolled reproduction impairs growth and survival during grow-out or poses environmental problems upon stocking in open waters. Present methods for sex control of fish involve either hormonal treatment or chromosome manipulation (Dunham, 1990). Both methods, though, present problems for practical application such as consumer reaction to hormone-treated or chromosome-manipulated fish, or require maintenance and periodical replenishment of sex-reversed broodstock. Thermal sex manipulation is a promising alternative to the methods above.

For practical application of TSD in aquaculture, combinations of temperature and duration of thermal manipulation for reliable production of specific sex ratios (all-female, all-male) will have to be established. As shown for O. bimaculatus (Strüsmann and Saitoh, unpublished data), it is a narrow step between functional all-male-producing temperatures and sterilization. Thus, temperatures and treatment duration for selective functional sex reversal or sterilization must be carefully chosen. It will be necessary to optimize other conditions that promote survival during thermal stress and to reduce thermal treatment duration. Wherever the desirable sex is obtained at the lower temperatures, it will be necessary to minimize the duration of thermal treatments also because low temperatures cause depression of growth rates. Finally, growth rates are likely to differ between hatcheries even when temperature is kept constant, and thus criteria to determine the end-point of treatments must be worked out.

Temperature control, even if not sufficient by itself, may still be needed for successful manipulation of sex by other methods. As shown in Table 1, putative monoecious broods produced by gynogenesis or from sex reversed parents often present varied proportions of the opposite sex, which may depend on rearing temperature as well as on parental genotype. Temperature also seems to act synergistically with exogenous steroid hormones during hormonal sex manipulation. Not surprisingly, the temperatures that potentiated the action of androgen hormones coincided with those that favored the formation of males during thermal sex manipulation, e.g. high temperature in C. intestinalis (Fujikawa, 1993; conf. Table 1). Also, complete masculinization of carp was only possible under high temperature (Nagy et al., 1981), a condition that favors male formation in other cyprinids (Table 1).

Concluding remarks

Information reviewed herein indicates that thermal liability of gonadal sex and sensitivity of germ cells to temperature are not unique to a certain group of fishes, nor do they seem to be exclusive attributes of species which adaptively take advantage of environmental sex determination. Many cases of unexpected sex ratios from crosses with otherwise genetically sex-determined species reported in the literature may have an environmental basis, either through direct thermally induced sex reversal of part of the progeny or indirectly through use of maternally sex-reversed parents.

The seemingly widespread occurrence of TSD and perhaps temperature-dependent sterilization among fishes ought to have important implications for the production of monoecious and sterile fish in aquaculture. Practical use will require that exposure to extreme temperatures is limited to a minimum, e.g. the period encompassing sex differentiation, in order to maintain adequate survival and growth rates. Externally visualized developmental events
that are concomitant with gonadal sex differentiation may constitute cues to determine the end-point of thermal treatments, and warrant further study. These external indicators may prove helpful especially in circumstances where substantial differences in growth rates between hatcheries (or trials) are expected. Temperature control, if not sufficient by itself for sex manipulation in some species, may be complementary to other sex control techniques such as hormone treatment and chromosome manipulation.

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References


TEMPERATURE SEX DETERMINATION IN TWO TILAPIA, OREOCHEIMIS NILOTICUS AND THE RED TILAPIA (RED FLORIDA STRAIN): EFFECT OF HIGH OR LOW TEMPERATURE.

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Summary

Very few studies have evaluated the role of external factors on sex determination in gonochoristic fish species. In tilapia, our recent results clearly demonstrate that high temperature has a strong effect on the Oreochromis niloticus sex-ratio. In reptiles and amphibians, 3 different patterns of temperature sex determination (TSD) have been described according to the sex produced at high and low temperature. In order to define this pattern in the tilapia, Oreochromis niloticus, we compared the effects of low and high temperature on the sex-ratio of standard or genetically female progenies. High temperatures (34-36°C) significantly increased the proportion of males (extreme percentages = 69-91% males) but low temperatures (19-23°C) did not affect the sex-ratio. Furthermore, we also investigated TSD in red tilapia of the Red Florida strain (taken as a gene pool of 2 MH and 2 FH species). In this red tilapia, treatments at 36°C also significantly increased the percentage of males in most progenies (extreme percentages = 60-97%). These results suggest that TSD could exist in several tilapia species, with strong genotype-temperature interactions. The pattern of this TSD resembles that of the other thermosensitive fish, Menidia menidia.

Introduction

Since the first evidence of Temperature Sex Determination (TSD) in a gonochoristic fish, Menidia menidia (Conover and Kynard, 1981), very few studies have focused on other species (Sullivan and Schultz, 1986; Baroiller et al., 1993; 1995). In Oreochromis niloticus, sex is determined by genetic factors, temperature, and genotype/temperature interactions: at standard temperatures, sex-ratios generally tally with the sexual genotype, whereas high temperatures applied during a critical period may affect the gonadal sex differentiation (Baroiller et al., 1993; 1995). In the fish, M. menidia, as in many thermosensitive reptile and amphibian species, low and high temperatures oppositely affect the sex-ratio (Conover and Kynard, 1981; Spotila et al., 1994). As wild tilapia may experience a wide range of temperatures, we have tested the effects of low and high temperature on the Oreochromis niloticus sex-ratio. Moreover, as different

Oreochromis niloticus progenies tend to vary as to the the level of TSD (Baroiller et al., 1993; 1995), we have examined some characteristics of these variations.

Materials and methods

Standard O. niloticus ("Bouakké strain") and red tilapia (Red Florida strain) or genetically all-female O. niloticus progenies were subjected for 28 days to various temperature treatments during the thermosensitive period (Baroiller et al., 1993, 1995). Variation in thermosensitivity was examined in 2-3 successive progenies from 5 different O. niloticus breeding pairs. The inheritance of thermosensitivity was examined in 2 temperature sex-reversed males. In both experiments, progenies were divided in 2 groups and exposed to 27 and 36°C. Five red tilapia progenies from different breeding pairs were temperature-treated as described above for O. niloticus. Sex-ratios were determined on all fish by microscopic examination of gonad squashes, 90-120 days post-fertilization (PF) when the histological characteristics of male or female differentiation are already in place (Baroiller, 1988). Survival rates were not significantly affected by high or low temperature treatment. The Chi-square test (p<0.05) was used to compare the sex-ratios.

Results

Pattern of temperature sex determination

![Figure 1: Effect of various temperatures on O. niloticus sex-ratio](image)

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In both standard and genetically female *O. niloticus* progenies, high temperatures (34-36°C) significantly increased the proportion of males whereas low temperatures (10-23°C) did not affect the sex-ratio.

**Variations and inheritance of thermostensitivity:**

There was no significant difference in the response to treatment at a given temperature between replicate samples or among successive progenies issued from a same breeding pair. The progenies of 3 breeding pairs (I, II and III) responded to high temperature treatment by a significant increase in the proportion of males. In contrast, the progenies of the breeding pairs IV and V were insensitive to temperature treatment.

![Figure 2: Response of the sex-ratio to treatment at 27 or 36°C in several successive progenies (1-3) from 5 different *O. niloticus* breeding pairs (I-V).](image)

Temperature sex-reversed males for the inheritance experiment were obtained by the following procedure.

![Figure 3: Inheritance of thermostensitivity in the progenies of 2 temperature sex-reversed males of *O. niloticus*. Progenies 7 and 8 have been subjected to treatments at 36°C.](image)

Temperature treatment was applied to a standard progeny. In the 36°C treated group, the percentage of males obtained was 78% (Fig. 3a). Eight of these males were raised to maturity. By analysing the sex-ratio in the progenies of these 8 males it was possible to identify the temperature sex-reversed males. 4 males sired all-female progenies (Fig. 3c), whereas balanced sex-ratios were obtained from the other 4 males (Fig. 3b). Progenies of 2 temperature sex-reversed males which were subjected to 36°C yielded significantly higher proportion of males (57.4-68.7%) than in the groups at 27°C (Fig. 3c).

**Effect of temperature in red tilapia**

Here again, treatment at 36°C significantly increased the percentage of males in most of the progenies. As in *O. niloticus*, there was a strong parental influence on the response to high temperature-treatment.

![Figure 4: Response of the sex-ratio to treatment at 36°C among the progenies of different red tilapia breeding pairs (with replicate samples).](image)

**Discussion**

*O. niloticus* sex-ratios are governed by a female homogamy and modified by high temperatures. At low temperatures, the sex-ratios usually tally with the sexual genotype. Elevated temperatures result in predominantly male sex-ratios but never in all-male populations. The highest percentage of males recorded for *O. niloticus* was 92%, for the red tilapia, it was 98.3%.
In both tilapia species, strong genotype-temperature interactions are suggested:
- progenies from different breeding pairs vary as to their thermosensitivity (from 0 to 98.3% males following a same 36°C treatment).
- similar sex-ratios are observed among successive progenies of a same breeding pair when treated at a given temperature.
- temperature sex-reversed males can sire thermosensitive progeny.

Red tilapia of the Red Florida strain was originally obtained by crossing a mutant red O. mossambicus with 3 other species, O. hornorum, O. niloticus, O. aureus. The existence of TSD in red tilapia may simply reflect the O. niloticus contribution to the genome of this tilapia. It might also mean that such an effect exists in all four species from which it derives. This possibility deserves further study.

The O. niloticus TSD resembles that of another thermosensitive gonochoristic fish, the Atlantic sides Menidia menidia, however, in the latter species which has major sex-determining genes but no detected sex chromosomes, low temperatures produce mostly female offsprings (Conover et al., 1992).

In thermosensitive reptiles and amphibians, genotype-temperature interactions are generally not reported (Dournon et al., 1990).

In nature, wild tilapia may well encounter masculinizing temperatures during the critical thermosensitive period. However, the biological significance of TSD and the underlying physiological mechanisms remain to be elucidated. TSD may be more widespread than recognized.

References


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SALMON GnRH GENE EXPRESSION FOLLOWING PHOTOPERIOD MANIPULATION IN PRECOCIOUS MALE MASU SALMON

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Summary

In spring, a large individual variation appeared in the number of sGnRH neurons expressing sGnRH mRNA in the ventral telencephalon (VT) and the preoptic area (POA) of underyearling male masu salmon. Numbers showed positive correlation with GSI, although GSI was very low. This suggests that development of sGnRH neurons in the VT and POA is involved in the determination of future precocity.

In early summer, precocious maturation of underyearling male masu salmon was accelerated when fish were transferred from natural photoperiod to 8L16D, compared to fish transferred to 16L8D. Spermatiation was observed in August in the former, and in September in the latter. The number of sGnRH neurons expressing sGnRH mRNA in the VT and POA increased in August in the 8L16D group, whereas numbers increased in September in the 16L8D group. These results indicate that sGnRH synthesis is accelerated by decreasing daylength, and activated sGnRH synthesis is involved in the acceleration of precocious maturation. This may be the first demonstration that photoperiod regulates sGnRH neuronal activity in teleost fish.

Introduction

It has been known that male salmonid fishes precociously mature. Despite their interesting life history, mechanisms of precocious maturation have not yet been clarified. In this paper, we used masu salmon Oncorhynchus masou as an experimental fish, and examined how precocious maturation is regulated. Precocious maturation occurs in autumn in underyearling male masu salmon which show rapid growth, suggesting that precocious maturation is controlled by somatic growth and photoperiod changes.

Since gonadal maturation of salmonids is controlled by salmon type gonadotropin-releasing hormone (sGnRH) via stimulation of pituitary gonadotropin synthesis and release, sGnRH is considered to be involved in the regulation of precocious maturation (Amano et al. 1991). In a previous paper (Amano et al. 1994), we reported that when underyearling males are transferred from natural photoperiod to 8L16D or 16L8D in June, precocious maturation is accelerated in the 8L16D group with rapid increase in pituitary sGnRH content, suggesting that decreasing daylength accelerates sGnRH gene expression. Recent analysis of the nucleotide sequence of the masu salmon sGnRH cDNA (Suzuki et al. 1992) has made it possible to investigate sGnRH synthetic activity by using in situ hybridization techniques.

In a previous experiment using underyearling males in June (Amano et al. 1993), there was no difference in the appearance rates of precocious males between the 8L16D and 16L8D groups. This result suggests that future precocity is determined before June. In the experiment, sGnRH was also suggested to be involved in the determination of precocious maturation.

Therefore, in this paper we aimed to clarify the relationship between brain sGnRH gene expression and the regulation of precocious maturation. Two experiments were carried out at the Nikko Branch, National Research Institute of Aquaculture. Masu salmon used were offspring of wild fish which had migrated to the Shiribetsu River in Hokkaido.

Determination of future precocity and sGnRH gene expression in spring

Underyearling males which were reared under natural photoperiod and 9-10°C were sampled on April 16, May 7 and June 11. On the day of autopsy, fish were randomly selected and anesthetized in ethyl-p-amino benzoate (0.05%). For in situ hybridization, brains were removed rapidly and were fixed with 4% paraformaldehyde and 1% picric acid in 50 mM phosphate buffer (pH 7.3) at 4°C. In situ hybridization (ISH) was carried out according to the method previously reported (Amano et al. 1994). Testes were fixed with Bouin's solution and weight was measured for the calculation of GSI.

Body length and weight gradually increased from April through June. Mean GSIs were 0.060%, 0.043% and 0.143% in April, May and June, respectively; they increased significantly in
June. In June, gonadal weight and GSI showed wider distribution than in April and May. Proliferation of spermatogonia was not observed in all individuals in April and May. In June, proliferation occurred in fish which had relatively high GSI in June.

The number of neurons expressing sGnRH mRNA in the VT + POA increased in May and remained at the same level in June. Changes in total silver grains in the VT + POA showed a similar pattern as observed in the number of neurons expressing sGnRH mRNA. The number of neurons and total silver grains per individual fish showed wide distribution in May and June. There were positive correlations between GSI and the number of neurons expressing sGnRH mRNA in the VT + POA in April and June. Positive correlations were also observed between GSI and total silver grains in the VT + POA.

Effects of photoperiod on acceleration of precocious maturation and sGnRH gene expression in summer

In June, 320 underyearling fish, including males and females, were randomly selected from a stock reared under natural photoperiod and 9-10°C. They were then transferred to short photoperiod (8L16D) or long photoperiod (16L8D) conditions (160 fish each). Tissues were sampled on June 23 (initial), July 23, August 19 and September 28. Sampling procedures were as described above. In situ hybridization was carried out according to the method previously reported (Amano et al. 1994). Sex and gonadal stages were checked and only precocious males were used as material. Precocious males were easily distinguishable by increased GSI at all periods except at the beginning in June.

In the 8L16D group, GSI increased in July, remained at high levels in August, and then decreased in September. Spermatization was observed in August. In the 16L8D group, GSI increased from August and attained a peak in September, when spermatization was observed. In July, GSI was significantly higher in the 8L16D group than in the 16L8D group. On the other hand, GSI was significantly higher in the 16L8D group than in the 8L16D group in September (Fig. 1).

In the 8L16D group, the number of neurons expressing sGnRH mRNA rapidly increased in the VT and POA until August. In the 16L8D group, numbers were unchanged until August, increasing in September. In August, the number of ISH-positive neurons in both areas were significantly greater in the 8L16D group than in the 16L8D group (Fig. 1). There were no marked changes in the number of neurons expressing sGnRH mRNA in the terminal nerve ganglion and the olfactory bulbs in either photoperiodic groups during gonadal maturation. There were no significant changes in the number of silver grains per neuron in all brain regions, although numbers tended to decrease in the olfactory bulbs in both photoperiodic groups.

![Graph showing changes in GSI and the number of neurons expressing sGnRH mRNA in the VT + POA in underyearling male masu salmon following photoperiod changes.](image)

**Fig. 1** Changes in GSI and the number of neurons expressing sGnRH mRNA in the VT + POA in underyearling male masu salmon following photoperiod changes.

+ indicates that spermatization was observed. In each group, means indicated by differing letters, for example a and b, differ significantly (p<0.05). ** (p<0.01) and *** (p<0.001) indicate significant difference between the short and long photoperiod groups in each month.

**Discussion**

The number of sGnRH neurons in the VT + POA increased in May, one month earlier than the
increase in GSI. Positive correlations between GSI and the number of sGNRH neurons were observed in April and June. In June, precocious males became distinguishable by their increased GSI. Taken together, it is possible that the development of sGNRH neurons in the VT and POA is directly related to the determination of future precocity of male masu salmon, suggesting that the brain-pituitary-gonadal axis is established.

Precocious maturation and the activation of sGNRH synthesis in the VT and POA were coincidently accelerated by changing photoperiod from natural conditions to 8L:16D in June, suggesting that the following accelerating process exists. Decreased daylength accelerated an increase in the number of sGNRH neurons expressing sGNRH mRNA in both areas, and synthesized sGNRH was transported to the pituitary. Accumulated sGNRH in the pituitary (Amano et al., 1994) stimulated GTH synthesis and release, and secreted GTH accelerated precocious maturation. This may be the first demonstration that photoperiod regulates sGNRH neuronal activity in teleost fish.

Long photoperiod delayed, but did not suppress precocious maturation of undercarng males. Precocious males were expected to be destined for sexual maturity in May prior to the commencement of the experiment (Amano et al., 1993). Once the brain-pituitary-gonadal axis becomes active, gonadal maturation may proceed irrespective of photoperiod. Thus, changes in photoperiod from long to short daylength accelerates gonadal maturation via activation of sGNRH neurons in the VT and POA possibly through a photoperiodic signal input system; long photoperiod only delays the onset of sGNRH neuron activation and hence gonadal maturation.

It is unlikely that sGNRH neurons in the terminal nerve ganglion are involved in testicular maturation through GTH synthesis and release. sGNRH neurons in the terminal nerve ganglion are considered to have a neuromodulatory function in the brain of dwarf gourami, Colisa lalia (Oka and Ichikawa, 1990; Oka 1992), and goldfish, Carassius auratus (Kobayashi et al., 1992; 1994). Such may also be the case in masu salmon. sGNRH neurons in the olfactory bulbs also showed no remarkable changes during photoperiod manipulation, indicating that they are not involved in gonadal maturation.

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HOW DO PHOTOPERIOD, THE PINEAL GLAND, MELATONIN, AND CIRCADIAN RHYTHMS INTERACT TO CO-ORDINATE SEASONAL REPRODUCTION IN SALMONID FISH?


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Introduction

It is now well established that the principal environmental determinant of the timing of seasonal breeding in rainbow trout is the ambient photoperiod with only minimal influence imparted by temperature (Bromage et al., 1993). Advances in spawning time are elicited by long days early in the yearly cycle of reproduction particularly if followed later by short days; by contrast delays in spawning are brought about by constant short or by short and then long daylengths.

Although the influences of photoperiod are known to be mediated by the hypothalamic-pituitary-gonadal axis, with alterations in daylength being paralleled by changes in the timing of onset and patterns of GTH and other hormones (Bromage et al., 1982), many questions remain regarding the mechanisms by which information on daily and seasonally-changing daylength is perceived and transmitted by the fish and in turn how this influences the neuroendocrine axis. In mammals the pineal hormone melatonin has been shown to be involved. However, it is not known whether melatonin has a similar role in fish.

A further complication is that the overall process of reproduction in salmonid fish is now thought to be controlled by an endogenous circannual rhythm or "clock" (Bromage et al., 1993). Although this rhythm exhibits many of the features of other biological clocks, its level of influence on the photoperiodic response and in particular with the putative functions of melatonin remains unclear. Aspects of these questions are further considered in the present study.

Results

(i) Photoperiod and Melatonin Secretion: Two series of experiments were conducted. In the first, a group of potential 52 Atlantic salmon parr were maintained from hatching under a natural photoperiod (Lat. 52°N). On 15 Sept. (LD 12:5:11:5) serum samples were taken at 3hr intervals, over a 30hr period, for RIA of melatonin (Randall et al., 1995). Fig 1 shows that as light levels fell at dusk, melatonin values increased and remained elevated (>250pg/ml) throughout the dark period. By contrast at dawn, melatonin values rapidly decreased and were maintained at basal levels (approx 90pg/ml) throughout the light period.

In the second experiment, 3 groups of 2+ year old Atlantic salmon post-smolts were acclimated for 2-3 months to constant photoperiods of LD 12:12, LD 16:8 or LD 20:4 and serum samples taken at 2hr intervals over 30hr periods for melatonin assay. Under each regime, melatonin levels were significantly (p<0.001) elevated throughout the dark periods (Fig 2); the duration of the raised melatonin values exactly reflecting night-length.

(ii) Seasonal Patterns of Melatonin Secretion: Plasma samples were taken at 3hr intervals over 27hr sampling periods, at approximately monthly intervals, from groups of potential 52 salmon parr exposed to a natural photoperiod (56°N). Measurements of melatonin showed that as daylength increased in the Spring, up to the Summer Solstice, then the duration of raised melatonin levels each day gradually decreased and was shortest on the longest day (Fig 3). Conversely, as daylength decreased in the Autumn/Fall up to the shortest day in December, the duration of raised melatonin progressively increased.

(iii) Effects of Pinealectomy and Melatonin Implantation on Spawning Time: Several pinealectomy (PNX) experiments have been carried out with pre-pubertal trout and salmon. Blood samples taken mid-way through light and dark periods in PNX and intact and sham-PNX control fish, at regular intervals starting 3 weeks after surgery and continuing until after spawning, confirmed that on all occasions controls had normal diel patterns of melatonin, high at night and basal during the day, whereas PNX fish had basal (daytime) levels

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Fig 1: Diel melatonin profile (LD12.5:11.5) throughout the day and night. Weekly examinations of fish to determine the times of ovulation revealed significant delays in spawning in PNX fish compared to controls. Fig 4 shows the delay in spawning following PNX at the Summer Solstice of 2 year old rainbow trout.

For the implantation experiment two groups of salmon smolts were transferred to sea water on 15 March. On 18 April the following year, half of the fish received intramuscular implants of melatonin, which had previously been shown to provide supraphysiological levels of melatonin (approx 1500 pg/ml) for periods up to a year (Randall & Bronage, unpublished). Weekly examinations of the fish showed no significant differences in spawning times of the implanted and control fish although there appeared to be a loss in synchrony in the implanted group, possibly indicative of a "free-running" rhythm. The absence of a clear response to continuous melatonin infusion may be due to the requirement for periods of low or minimal melatonin levels for proper transmission of the photoperiodic message.

(iv) Endogenous Rhythms of Melatonin Secretion: Groups of salmon S1 post-smolts were subjected to the following photoperiod regimes: (1) a simulated natural seasonal light cycle, 6 months out-of-phase with the ambient photoperiod, (2) a simulated natural seasonal cycle until 21 December with the daylength on that day (i.e. short day) then held constant (Winter Solstice-hold) and (3) a stimulated natural seasonal cycle until 21 June and then

Fig 2: Diel serum melatonin under LD12:12, LD16:8 and LD20:4.
Conclusions

(1) The results show clearly that under a range of seasonally-changing and constant photoperiods, melatonin levels accurately reflect the length of the period of darkness. It is suggested that the diel and seasonally-changing patterns of circulating melatonin encodes information on both daily and calendar time which is then used to time daily and seasonal events.

(2) The ability of alterations in photoperiod to bring about advances and delays in spawning and in parallel to produce modifications in profile of melatonin secretion suggests that melatonin is a mediator of the photo-periodic control of reproduction.

(3) Pinealectomised fish did not exhibit any diel changes in circulating melatonin. Pinealectomy at the time of the Summer Solstice produced a delay in spawning, compared to controls suggesting that the pineal (and melatonin) is necessary for the entrainment of reproduction which, in part, is normally brought about by the decreasing daylengths (ie. increased daily duration of melatonin secretion) of Autumn.

(4) Implants of melatonin failed to modify spawning time possibly because alternating periods of high and low melatonin are required for successful transmission of information on daylength.

(5) The consistent representation of night length by the duration of melatonin increase under both the out-of-phase seasonal and Solstice-hold photoperiods indicates that there is no endogenous circannual influence on melatonin secretion.

(6) It is concluded that the endogenous rhythm(s) which control reproduction in salmonid fish must be acting "down-line" from the information provided by melatonin on daily and calendar time.


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PHOTOPERIOD CONTROLS THE TIMING OF REPRODUCTION IN ATLANTIC COD (GADUS MORHUA).

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Summary

Juvenile Atlantic cod (Gadus morhua) were submitted to four different photoperiod treatments, designed to advance or delay spawning time. The results clearly show that photoperiod manipulation can be used to alter and control the time of first and subsequent spawnings in Atlantic cod of both sexes.

Introduction

The Atlantic cod is a teleost species of great commercial importance to the fishing nations surrounding the North Atlantic. Methods for successful cultivation of cod exist, and the depletion of several wild cod stocks has created a renewed interest for aquaculture of this species. A stable, continuous supply of juveniles is crucial in order to achieve a commercially viable cod production. Since the cod is a seasonal breeder, manipulation of spawning time is an important tool for obtaining a year-round production of fry.

The present study describes the successful manipulation of spawning time in male and female Atlantic cod, by treatment with compressed or extended annual photoperiod cycles. Plasma levels of the sex steroids estradiol-17β and testosterone were monitored in females, and plasma levels of testosterone were monitored in males.

Materials and methods

Experimental design

Juvenile, one year old, Atlantic cod of both sexes, with an average weight of 310 g, were randomly divided in four groups of 150 individuals. The groups were kept in circular indoor tanks (3m diameter), surrounded by light-proof black plastic and with a fluorescent daylight tube, connected to a time switch, as light source. On July 1 1992, the experiment was started, by submitting the four groups to four different annual photoperiod cycles:

1. ADV6 - first annual photoperiod cycle compressed to six months, second annual photoperiod cycle twelve months.
2. ADV3 - first annual photoperiod cycle compressed to nine months, second annual photoperiod cycle twelve months.
3. DEL - first annual photoperiod cycle extended to eighteen months, second annual photoperiod cycle twelve months.
4. NORM - first and second annual photoperiod cycles twelve months.

Each month, the fish were checked for signs of sexual maturation, i.e. running eggs and milt and blood samples were taken from twenty randomly chosen individuals. To reduce sampling stress, no individual was sampled two months in a row. The blood samples were immediately centrifuged and the plasma frozen on N₂(1). The frozen plasma samples were stored at -20°C until analysis.
Steroid analysis

Plasma levels of estradiol-17β and testosterone were analysed by previously validated radioimmunoassays (Methven et al., 1992). Antisera against estradiol-17β and testosterone were purchased from ICN Immunobiologicals Inc., reference standards were purchased from Sigma Chemical Co. and tritium labelled steroids were obtained from Amersham.

Results

Spawning time

In group ADV6, fish with running eggs and milt were first detected in January and February 1993. In July 1993, running males were again detected. The following month, spawning behaviour was observed in the tank and at sampling, all females had running eggs. Eggs and milt was detected until October, while only mature males were found in November.

In group ADV3, running males were first detected in January 1993. In February and March, both male and female fish were mature. In April, only mature males were found. Running males were again detected in this group in November and December 1993, while both females and males were mature in January 1994.

In group DEL, running males were found from February to August 1993, while mature fish of both sexes were found from March until July this year. In 1994, the fish spawned from September to November, and mature males were found until December.

In the control group, NORM, mature males were found from January to May both in 1993 and in 1994. Females had running eggs from February to May in 1993 and from February to April in 1994.

Fig. 1. Plasma levels of estradiol-17β and testosterone in female Atlantic cod, held at four different annual photoperiod cycles. Filled squares: estradiol-17β; open squares: testosterone.
Plasma steroid hormone levels

Plasma levels of estradiol-17β and testosterone in female cod, and of testosterone in male cod, are shown in Figs. 1 and 2, respectively. The highest plasma steroid levels were detected shortly before or during spawning in both female and male cod.

Discussion

Photoperiod manipulation is a powerful tool for controlling the timing of reproduction in Atlantic cod, as shown by the results of the present study. Steroid hormone levels were similar and in accordance with previous investigations (Kjesbu, Kryvi and Norberg, unpublished results) in the various groups. The underlying physiological mechanisms enabling different groups of cod to mature and spawn twice in one year, or extend their spawning period from three to five months in response to different photoperiod treatment, remain largely unknown and merit further investigation.

References


Fig. 2. Plasma levels of testosterone in male Atlantic cod held at four different annual photoperiod cycles.
EFFECTS OF SPRING TEMPERATURE AND FEEDING REGIME ON SEXUAL MATURATION IN ATLANTIC SALMON (SALMO SALAR L.) MALE PARR

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Summary

Food restriction in spring had a significant negative effect on the incidence of maturation in male salmon parr, but maturation seems to be suppressed in only a small part of the experimental populations. Even males with very low or negative growth rate in June matured. Water temperature in spring seems, however, to have a more general effect on the onset of rapid gonadal growth in late June; low water temperature during May and June inhibited sexual maturation in most males.

Introduction

Sexual maturation in male Atlantic salmon (Salmo salar L.) parr is common under both natural and hatchery conditions. The probability of maturation in male parr aged 1+ yrs is positively related to its size at the end of the first summer under both wild (Myers et al. 1986) and experimental conditions (Berglund 1992; Simpson 1992). Furthermore, it has been found that the opportunity for growth, in spring, before the start of rapid gonadal growth in early summer, affects the proportion of mature parr (Rowe and Thorpe 1990; Berglund 1992; Prevost et al. 1992).

The preliminary “decision” of a male parr to mature at age 1+ seems to be based on its size after the first summer (Myers et al. 1986; Berglund 1992; Simpson 1992). The accomplishment of this decision seems, however, to depend on the growth rate in spring the second summer. It has been suggested that rapid spring growth and accumulation of fat are necessary for the onset of rapid gonadal growth in early summer. The present paper reports on the effects feeding opportunity and water temperature in spring on the probability of sexual maturation in experimental groups of 1+ male salmon parr.

One-year-old Atlantic salmon parr of Baltic origin, belonging to the Skellefteälven (Skellefte River stock, 64°45'N, 21°E), were used in this study. Fish were raised from eggs under standard hatchery conditions with through-flowing river water at ambient temperature and under natural photoperiod. Experiments were performed in 1992, 1993, and 1994. During the experiments groups of fish were subjected to a treatment, feeding or temperature regime, from early May (5–9, depending on year) to late June (23–27), after that they were reared at ambient temperature and fed at excess until the incidence of sexual maturation were determined in September. In all experiments 150–220 fish, selected at random, were used in each treatment group. Experimental groups were kept in 4-m³ tangential-flow tanks and fed commercial fish-feed pellets dispensed by automatic feeders. All fish were individually tagged with passive integrated transponder (PIT) tags.

In 1992, effects of individual size and feeding opportunity in spring on the size-specific probability of sexual maturation were studied (see Berglund 1995 for details). Fish were subjected to three different feeding regimes: (1) controls fed at excess, (2) alternate starvation (three days)/excess feeding (two days), and (3) restricted (ca. 40% of recommended) daily ration, in May and June. Fish were reared at ambient temperature, which increased rapidly from 3° C in early May to about 16° C in mid June. There were two replicate tanks with 220 fish in each regime. In 1993, a preliminary temperature experiment with two temperature regimes was performed. In the ambient regime (two tanks) the temperature rose from 5° C in mid May to 12° C in mid June, and in the accelerated regime (one tank) from 4° C to 16° C. In 1994, three temperature regimes (two tanks in each) were applied. All regimes started at 3° C on 9 May, and reached 11, 13, and 16° C, respectively, by mid June. All fish were measured at the start and at the end of the treatment periods, and their individual increase in weight and condition factor (100(weight/length³)) were calculated.

Results

In 1992, the proportion of mature males decreased with decreasing feeding opportunity in May–June (Table 1, Berglund 1995). The mean proportions of mature males in the restricted regimes were significantly reduced compared with the control regime.

Generally, the probability of maturation increased with increasing body length, reached a maximum, and then decreased for the largest males (Fig. 1). By logistic regression it was established that the effect of

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length of yearling parr on the probability of maturation differed among treatments (Log Likelihood ratio test, p < 0.001). The decrease in maturation rate among fish larger than 9 cm, was due to an increasing frequency of one-year-old smolts, which did not mature sexually, with increasing size (Berglund 1995). Food restriction during June or May and June shifted the peak in maturation rate towards larger fish and suppressed maturation in males smaller than ca. 10 cm in length; males larger than 10 cm showed similar or higher maturation rate compared with controls (Fig. 1).

Table 1. Proportion of mature males and growth in weight in groups of salmon parr subjected to different feeding and temperature regimes in May and June.

<table>
<thead>
<tr>
<th>Year/Regime</th>
<th>Proportion mature (%)</th>
<th>Growthb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1992 Feeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>61</td>
<td>0.88</td>
</tr>
<tr>
<td>Starvation</td>
<td>46</td>
<td>0.10</td>
</tr>
<tr>
<td>Low ration</td>
<td>35</td>
<td>0.19</td>
</tr>
<tr>
<td>1993 Temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>54</td>
<td>1.01</td>
</tr>
<tr>
<td>Low</td>
<td>13</td>
<td>0.81</td>
</tr>
<tr>
<td>1994 Temperature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>37</td>
<td>1.07</td>
</tr>
<tr>
<td>Medium</td>
<td>15</td>
<td>0.87</td>
</tr>
<tr>
<td>Low</td>
<td>5</td>
<td>0.69</td>
</tr>
</tbody>
</table>

a) Within year, control or high temperature treatments differed from the other treatments by χ²-test, p<0.01. 
b) ln(weight at end of treatment) - ln(weight at start of treatment). Data represent fish with a length of 7 to10 cm in early May.

Maturing males grew faster than non maturing males in the low ration regime, (ANCOVA, F 1,136 = 9.69, p < 0.01), but growth rates were similar in the other regimes. It should be noted that 10 of the 71 maturing males in the low ration regime decreased in weight during June.

In 1993 and 1994, the proportion of mature males was positively related to the temperature increase during May and June (Table 1, Berglund unpublished). Furthermore, maturation rate seems to decline more rapidly with decreasing growth in May and June if the water temperature is kept at a low level than if food ration is restricted.

Discussion

Food restriction in spring had a significant negative effect on the incidence of maturation in male salmon parr in this study, but maturation seems to be suppressed in only a small part of the experimental populations. Water temperature in spring seems, however, to have a more general effect on the onset of rapid gonadal growth in late June; low water temperature in May and June inhibited sexual maturation in most males.

Fig. 1. Logistic regression lines showing the relationship between the probability of sexual maturation in male salmon parr at age 1+ in relation to size at age 1 year. Fish were subjected to three different feeding regimes in May and June: controls at excess, alternate starvation/feeding, and low (maintenance) ration. The general form of the regression equation was: Probability of maturation = 1/(1+exp(-β1(length) + β2(length²))); (b) Absolute change in maturation rate for groups on restricted food. The change was calculated as: logistic regression equation for restricted group - logistic regression equation of control. Reference line at change = 0 is given.

The occurrence of maturing males among starved fish with very low growth rate in June, and in poor condition by the end of June, implies that high growth rate in June is not necessary for the onset of rapid gonadal growth in early summer. This is further
supported by the finding that slow temperature development in spring depressed maturation rate despite comparatively high growth rates.

Rowe et al. (1991), suggested that aromatization of testosterone in fat stores should be the physiological link between fat accumulation during spring and the initiation of gonadal growth in early summer. The above results, and the finding that no estrogens were formed from radiolabelled androstenedione in incubations of mesenteric fat tissue from male parr in mid June (Borg, Mayer, Berglund, and Lambert, unpublished data), makes the suggested mechanism unlikely.

The present findings suggest that water temperature in spring and early summer has stronger influence on maturation rate in male salmon parr than food availability and growth rate per se. It seems likely that the long-term growth, affecting size-at-age, of individuals, and the water temperature in spring and early summer are the most important factors explaining between-year variation in the proportion of mature male parr within salmon populations.

Acknowledgements

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References


APPLICATION OF REPRODUCTIVE PHYSIOLOGICAL TESTING TO UNDERSTAND THE MECHANISMS OF ENVIRONMENTAL ENDOCRINE DISRUPTORS

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Abstract

Assessment of the reproductive performance of fish is increasingly being used to evaluate the impacts of environmental disturbances. Reproductive physiologists are being called upon to provide quick answers to questions related to the identity and actions of compounds in complex mixtures, and the effectiveness of chemical process changes and remedial treatment regimes. At this time, there are no surrogates for long term experiments, although physiological testing of wild fish populations is providing a means of focusing attention to key components of the reproductive axis sensitive to environmental disturbances.

Introduction

There is growing awareness and concern that environmental contaminants can influence the endocrine system and thereby play a role in the developmental and reproductive problems observed in wildlife and human populations. The term endocrine disrupting chemicals (EDCs) has been used to categorize the broad suite of environmental compounds which affect endocrine function (Colborn et al. 1993). There is ongoing debate as to whether EDCs contribute to the increased incidence of breast cancer in women, or the increase in reproductive tract disorders in men and proposed progressive declines in human sperm counts (Colborn et al., 1993; Safe 1995).

A large number of EDCs have been shown to alter reproductive endocrine function in fish in field and laboratory studies (Table 1). For example, depressed levels of circulating steroids and altered reproductive function have been observed in fish collected downstream of pulp mills and sewage treatment plants, at sites contaminated with polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Johnson et. al. 1988; Munkittrick et al. 1992; Purdom et al. 1994; see review in McMaster et al. 1995a). Other alterations in reproductive function include smaller gonads and egg size, delayed sexual maturity, reduction or sex-reversal of secondary sexual characteristics, changes in sex ratios, and increased embryonic and early life stage mortality.

<table>
<thead>
<tr>
<th>Table 1. Some of the EDCs that affect reproductive function in fish.</th>
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<tbody>
<tr>
<td>Pesticides</td>
</tr>
<tr>
<td>Herbicides, Insecticides, Fungicides</td>
</tr>
<tr>
<td>Industrial and Synthetic Chemicals</td>
</tr>
<tr>
<td>Metals (Cd, Pb, Hg)</td>
</tr>
<tr>
<td>Persistent organochlorines (Dioxins, PCBs)</td>
</tr>
<tr>
<td>PAHs</td>
</tr>
<tr>
<td>Alkylphenols</td>
</tr>
<tr>
<td>Phthalates</td>
</tr>
<tr>
<td>Phytochemicals</td>
</tr>
<tr>
<td>Isoflavones, Sterols</td>
</tr>
</tbody>
</table>

While monitoring wild fish populations may provide the strongest measure of ecological relevance, rapid laboratory testing will be required to evaluate both the increasing number of suspected EDCs and the effectiveness of remediation techniques once problems are identified. There is also a growing awareness that existing, standard, toxicological test methods have not been effective in detecting the actions of EDCs. Standardized test methods to screen for the activity of EDCs, have to be established for fish. In vitro (receptor binding, steroid and vitellogenin production) and short term whole animal tests (plasma hormone levels, functional testing of the hypothalamo-pituitary-gonad axis) have been regularly used to assess the physiological mechanisms mediating reproductive processes. It is necessary to adapt the existing technology for:

a) use in evaluating the effects of EDCs, 
b) identification of EDCs present in complex environmental mixtures, and 
c) developing functional linkages between physiological changes and whole organism
effects.

This paper reviews some of the limitations of current in vivo and in vitro testing protocols for application in evaluating the effects of EDCs.

In Vivo Endocrine Responses

Many studies evaluating wild fish populations have included measurement of reproductive hormone levels. Our work downstream of a bleached kraft pulp mill showed that white sucker (Catostomus commersoni) and lake whitefish (Coregonus clupeaformis) had depressed plasma sex steroid and GTH II levels which correlated with whole organism responses (e.g. gonad size, sexual maturity, secondary sexual characteristics) (Munloitt et al. 1992; Van Der Kraak et al. 1992). By comparison, longnose sucker (Catostomus catostomus) from this site had depressed hormone levels but no consequences at the whole organism level. These findings illustrate the difficulty in interpreting the effects of EDCs on reproductive function. Although the endocrine events which mediate reproductive processes are well understood, we have a limited understanding of which endocrine responses offer the most potential for prediction of whole animal and population level effects.

There is a lot of current interest in the measurement of vitellogenin levels as an indicator of exposure to EDCs with estrogenic activity (Purdom et al., 1994). While there is great public concern over the presence of female specific proteins in male fish, the consequences of vitellogenin production to the long term reproductive performance is unknown. The relevance of these changes needs to be carefully assessed in wild fish.

Laboratory exposures are useful for screening compounds and elucidating mechanisms, but often do not integrate all of the conditions and stressors present in a field environment. Although indications of changes in wild fish exposed to contaminants is the best measure of ecological relevance, artificial exposures of naïve fish to effluents in the laboratory or at field locations can be invaluable. These tests can provide critical information to aid in establishing cause and effect linkages and separating the confounding effects of environmental variables (altered habitat, temperature changes, etc.) in field studies.

Field caging studies using goldfish (Carassius auratus) have been successfully applied to our work on pulp mills (McMaster et al. 1995b) and by Sumpter’s group in England using rainbow trout (Oncorhyncus mykiss) downstream of sewage treatment lagoons (Purdom et al. 1994). Generally, these studies have been of short duration and the long term consequences of exposure, including multi-generational effects, have not been addressed. In mammals, the developing embryo tends to be the most sensitive to EDCs exposure (Colborn et al. 1993).

Testing the functional integrity of the pituitary gonadal axis in wild or laboratory exposed fish can be used to characterize the actions of EDCs (Van Der Kraak et al. 1992). Monitoring the response of fish to exogenous GnRH administration and in vitro incubation of gonadal tissue served to identify sites within the pituitary-gonadal axis altered by EDCs. Knowledge of the mechanisms underlying reproductive changes can serve to identify the impact points, which can focus follow up studies on relevant endpoints to identify or characterize the responsible stressors.

In Vitro Endocrine Responses

EDCs impact the reproductive process through a wide variety of mechanisms (Table 2). However, most of the work to date has focused on interactions with the estrogen receptor and induction of estrogen dependent responses. A diverse array of environmentally relevant chemicals including the phytosterols, non-ionic surfactants (nonylphenol), pesticides (o,p-DDT and DDE) and hydroxylated PCBs (White et al., 1994; Tremblay et al., this volume, P.Thomas unpublished) bind to the hepatic estrogen receptors of fish. Compared to 17β-estradiol, the receptor affinity of xenobiotics with estrogenic activity are often 4 to 6 orders of magnitude lower in activity. This has led some investigators to question whether interactions of xenoestrogens with the estrogen receptor account for the biological effects of these compounds (i.e. Safe, 1995). EDCs with estrogenic activity show significant differences in the relative potencies between in vitro (receptor binding) and in vivo bioassays (plasma steroid levels) which questions the predictive value of the in vitro test methods. Understanding the actions of EDCs with
estrogenic activity is further complicated by 17β-estradiol having multiple sites of action which may differ in sensitivity (Table 3). This calls into question which endpoint would be most predictive.

Table 2. Some of the mechanisms of action of EDCs.

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<thead>
<tr>
<th>Target</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain/Hypothalamus</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Pituitary</td>
<td>GnRH secretion</td>
</tr>
<tr>
<td>Liver</td>
<td>GTH secretion</td>
</tr>
<tr>
<td>Ovary</td>
<td>Steroid biosynthesis</td>
</tr>
<tr>
<td>Plasma</td>
<td>Carrier Proteins</td>
</tr>
</tbody>
</table>

Many in vitro test methods could serve as surrogates for whole animal testing for reproductive toxicity (e.g., receptor binding, mitogenic responses, hormone biosynthesis and metabolism). EDCs may exert their effects through more than one mechanism. For example, hydroxylated PCBs are estrogenic, but they also interact with the Ah receptor, which is responsible for turning on the synthesis of a variety of proteins, including hepatic P450 detoxification enzymes. The potential endocrine consequences of the interactions of the various disruptions is poorly understood.

Conclusion

There are no surrogates for whole animal testing, and laboratory testing lacks the integration provided by multi-generational exposures of wild organisms. Existing physiological testing protocols must be carefully integrated into studies of EDCs to provide insight into the mechanisms of disruption before predictive test methods can be developed.

References


