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Endocrine and environmental aspects of sex differentiation in fish

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Abstract. This paper reviews the current knowledge concerning the endocrine and environmental regulations of both gonadal sex differentiation in gonochoristic and sex inversion in hermaphroditic fish. Within the central nervous system, gonadotropins seem to play a role in triggering sex inversion in hermaphroditic fish. In gonochorists, although potentially active around this period, the hypothalamo-pituitary axis is probably not involved in triggering sex differentiation. Although steroids and steroidogenic enzymes are probably not the initial triggers of sex differentiation, new data, including molecular approaches, have confirmed that they are key physiological steps in the regulation of this process. Environmental factors can strongly influence sex differentiation and sex inversion in gonochoristic and hermaphroditic fish, respectively. The most important environmental determinant of sex would appear to be temperature in the former species, and social factors in the latter. Interactions between environmental factors and genotype have been suggested for both gonochoristic and hermaphroditic fish.

Key words. Teleost fish; sex differentiation; central nervous system; gonadal steroids; steroid enzymes; steroid receptors; gonadotropins; environmental factors.

Introduction

With over 20,000 species, the class of fishes exhibits a large variety of adaptation responses to match the vast array of existing ecological habitats. One of the most intriguing phenomena is probably the large number of reproductive strategies developed by these species. Sexuality represents an important aspect of these reproductive strategies: 'Members belonging to the class Pisces exemplify an almost complete range of various types of sexuality from synchronous hermaphroditism, protandrous and protogynous hermaphroditism, to gonochorism' [1]. The peculiar pattern of gonadal ontogeny in cyclostomes and teleosts, in which hermaphroditism occurs, has been suggested to be involved in this diversity [2]. The gonads of vertebrates which develop in the dorsolateral lining of the peritoneal cavity generally possess a double origin: the more laterally located cortical portion generates the ovary, whereas the medullary portion gives rise to the testis. In cyclostomes and teleosts, only a single primordium (cortex) appears to be involved in the ontogeny of both ovary and testis. This classical model requires further investigation using new embryological methods, such as the labelling of cell lines. Such methodologies have been used to determine the origin of primordial germ cells (PGCs), which appear to arise epigenetically in a manner very similar to that observed in higher vertebrate groups [3].

In addition to the diversity of sexuality in fishes, it is now well established that phenotypic sex in fish may depend on external factors, although the effect of such factors will differ from one species to the next [4, 5]. This plasticity of gonadal development in fish, which contrasts with the more stable patterns found in higher

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vertebrates, has given rise to a number of exciting questions concerning both its adaptive significance [6–9] and the underlying genetic and physiological regulations involved.

At this point, we must refer to Hayes's terminology which places emphasis on the difference between 'sex determination' and 'sex differentiation': the former expression designates mechanisms that direct sex differentiation, whereas the latter refers to the development of testes or ovaries from the undifferentiated or bipotential gonad [10]. In the present review, genetic sex determination (GSD) will be only briefly evoked.

In gonochoristic species presenting a simple heterogametic GSD model (XX/XY or ZW/ZZ systems), the possibility of hormonal sex inversion has been used to obtained genetic monosex male or female populations. Not only XX neomales or ZZ neofemales, but also new viable genotypes (YY males and WW females, by crossing XY neofemales with XY males or ZW neomales with ZW females, respectively) can be used as breeders for producing monosex populations [11–15]. These possibilities have relevant practical implications for fish farming [14–16], and the hormonal induction of sex inversion has now been extended to a large number of species [17]. In addition, genetic monosex populations are tremendously helpful in biological studies, as they allow research to be carried out on a large number of known-sex individuals for which the sex is known from fertilization, and thus before any detectable sign of sexual differentiation. Examples of such studies will be given in the following review concerning both the effects of external factors on sex determination and the physiological investigations of sex differentiation.

Several comprehensive reviews dealing with the morphological and histological description of sex differentiation [18] and sex inversion [4] have already been published. These aspects will therefore not be developed in the present paper in order that we may focus on two active fields of research for which a wealth of recent data has been obtained, namely the role of external factors and the involvement of endocrine factors in either gonochoristic sex differentiation or hermaphroditic sexual inversion. It should be noted, however, that the chronology of gonadal sex differentiation is highly variable from one species to the next, but also within a given species where the growth rate (and thus water temperature) is an important factor [19, 20]. In light of this, the time scale adopted for the kinetics of gonadal development is often expressed in terms of the age of larvae in days multiplied by degrees post-fertilization (PF) instead of only in days post-fertilization (dpf). The actual kinetics of gonadal differentiation should thus be determined prior to each new experiment where an accurate correlation between morphological features and physiological events is to be analyzed. Unfortunately, there is still a lack of definitive criteria for the detection of the very first discrete signs of differentiation [19]. Until now, the initiation of meiotic activity, shortly after active germ cell proliferation, has frequently been reported as being the first recognizable indication of ovarian differentiation. This is due to the precocious differentiation of female germ cells, which contrasts with the late appearance of the first meiotic prophase feature in future spermatocytes.

The present review will focus on the teleost superorder [4] which constitutes more than 90% of fish species. Concerning hermaphroditic species, the following terminology will be used: synchronous hermaphroditism (when testis and ovarian tissues are simultaneously active) versus successive hermaphroditism, including protandrous hermaphroditism—inversion of males into females—and protogynous hermaphroditism—inversion of females into males. In addition, in certain protogynous species males can differentiate directly into males ('primary males'), whereas others pass through a functional female stage ('secondary males'). This kind of phenomenon is referred to as 'diandry' and is rare in protandrous species ('digyny'), where primary and secondary females can be found [4].

Genetic sex determination

Although some species show morphologically well differentiated sex chromosomes [21], cytogenetic examinations are rarely helpful in identifying sex chromosomes in fish due to the low occurrence of heteromorphy [22, 23], even if some species show morphologically well differentiated sex chromosomes. Various methodologies, however, have been conclusive in the identification of GSD, and this can hardly be considered primitive [11, 24, 25]. The possibility of obtaining viable gynogenetic, androgenetic or hormonally inversed individuals offers original and powerful tools for such studies [26].

No simple model of GSD can be generalized in fish. Both male heterogamety (XX/XY), such as in several salmonids, and female heterogamety (ZZ/ZW), in *Gambusia*, have been reported. On the other hand, male and female heterogamety may be found even in the same species, as shown in the platyfish *Xiphophorus*, which could be an argument for a recent divergence of sex chromosomes [24, 27]. Other species-specific models have been proposed, incorporating multiple sex chromosomes, polygenic sex determination and autosomal influence [9, 12, 24, 28, 29]. Conversely, knowledge concerning the genetic determination of hermaphroditism remains limited [9, 28]. Molecular studies focussing on genes involved in fish GSD have been recently developed. SRY-type HMG (high mobility group) box (Sox) genes have been identified in some fish species [30–34]. However, and like other lower vertebrates, no functional sex-determining gene equivalent to SRY has been demonstrated. Only sex-specific probes have been obtained, all of them very specific to each species and even sometimes to a particular population of fish [21, 25, 35–39].

Endocrine regulation of sex differentiation and sex inversion

Involvement of the CNS in gonochoristic species

In gonochoristic species, very few studies have addressed the role of the central nervous system (CNS) in the process of gonadal sex differentiation. In addition, these studies have focused on the involvement of the hypothalamo-pituitary system via gonadotropin-releasing hormone (GnRH) and gonadotropin[1] hormones (GTHs). Using specific antibodies, GTH I immunoreactive cells have been found in the pituitary of the rainbow trout, *Oncorhynchus mykiss*, when mitosis of germ cells is first detected in the gonads [40], and localized in the pars proximal distalis (PPD) close to hatching (but no difference was detected in the pattern of expression of these cells in rainbow trout between male and female) [41]. In a closely related salmonid, the Coho salmon, *O. kisutch*, GTH I immunoreactive cells were detected 2 weeks after hatching [42]. When specifically searched for, GTH II was not detected during the time surrounding the sex differentiation period either in rainbow trout [40, 41] or Coho salmon [42]. Using nonspecific antibodies (either nonspecific according to species or type of GTH), GTH immunoreactive cells were detected in the PPD of rainbow trout when the gonads were thought to be still undifferentiated histologically [43]. In the common carp, *Cyprinus carpio*, GTH-containing cells were found in the pituitary gland of 3-week-old larvae [44]. GnRH immunoreactivity was only investigated in rainbow trout around the time of sex differentiation and was detected in both sexes in several brain regions, including those where GTH I was localized [41]. Growth hormone (GH) immunoreactivity in the pituitary gland of rainbow trout was detected at an even earlier stage than GTH I, namely slightly before the appearance of primordial germ cells (PGCs) [40]. It was suggested that GH may be involved in the sex differentiation process, as it is known that GH can act on regulation of steroidogenesis in adult salmonid gonads. In carp, a precocious sex differentiation was achieved following a treatment of 4-week-old larvae with homologous pituitary extracts [45]. This treatment resulted in a considerable increase in the PGC number, gonad size and GTH plasma levels as well as causing, in some fish, an acceleration of the appearance of histological features characteristic of female differentiation [45]. In light of the major role of GTHs in regulating gonadal steroidogenesis in adults [46, 47], Fitzpatrick and collaborators [48] have examined the potential effects of partially purified salmon GTH on gonads or gonadal-kidney explant steroidogenesis during sex differentiation in rainbow trout. GTH was shown to stimulate gonadal steroid production but only after histological gonadal differentiation. A much earlier stimulation of androstenedione[2] (Δ_4) production by the anterior kidney (interrenal) was also observed. Whether this production is significant with respect to gonadal differentiation in fish is not known, although a participation of the interrenal in the production of steroids potentially acting on gonad differentiation has been proposed in rainbow trout [49]. All these studies have demonstrated that the hypothalamo-pituitary axis is potentially active around the time of sex differentiation and that steroids can have a feedback effect on this axis in the same way as they do in adults [50]. Although a synthesis of GTH I in the pituitary seems to be clearly established around the time of gonadal differentiation at least in salmonids, it remains to be determined whether there is an active secretion.

Involvement of the CNS in hermaphroditic species

The potential involvement of the brain in triggering sex inversion was first investigated by Tang and collaborators in the protogynous ricefield eel, *Monopterus albus* [51]. In this study, female ricefield eels were induced for initiation of sex inversion with mammalian LH. LH activity of pituitary extracts was also found to be much higher during the female phase, decreasing in the transitional fish and very low in males. FSH activities were considered to be more or less similar in all the sexual stages [52]. These results, subsequently confirmed in the same species [53, 54], led to the hypothesis that a rise in GTH secretion in the female could trigger precocious sex inversion at least in protogynous species. Since these initial experiments, numerous studies have looked at the involvement of the brain in sex inversion of hermaphroditic species. Among protogynous species, tropical wrasses have been the subject of extensive exper iments. For instance, in the bluehead wrasse, *Thalassoma bifasciatum*, injection of females with human

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[1] We will rely in this review on the classical terminology for fish GTHs (GTH I and GTH II), although a new nomenclature has been proposed recently in fish. This new terminology proposes that GTH I (gonadotropin type I) be considered as FSH (follicular stimulating hormone) and GTH II as LH (luteinizing hormone). Unless stated otherwise in the article, we used the generic term GTH.

[2] Common names, abbreviations used and systematic names for the steroids cited in the text are given in table 1.

Table 1. Common and systematic names of the main steroids cited in the text with the abbreviations used.

Common names	Abbreviations	Systematic name
Estrogens (C_{18})	1	
Estradiol-17β	E_2	1,3,5,(10)-estratriene-3,17β-diol
Estrone	E_1	3-hydroxy-1,3,5(10)-estratriene-17-one
Ethynyl-estradiol	EE_2	1,3,5(10)-estratrien-17α-ethynyl-3,17β-diol
Androgens (C_{19})	1	
Androstenedione	$\Delta 4$	4-androstene-3,17-dione
Testosterone	T	17β-hydroxy-4-androsten-3-one
Dehydroepiandrosterone	DHEA	3β-hydroxy-5-androsten-17-one
5α-Dihydrotestosterone	DHT	17β-hydroxy-5α-androstan-3-one
11-Oxygenated androgens	1	
11β-Hydroxyandrostenedione	11βOHΔ_4	11β-hydroxy-4-androstene-3, 17-dione
11β-Hydroxytestosterone	11βOHT	11β,17β-dihydroxy-4-androsten-3-one
11-Ketotestosterone	11KT	17β-hydroxy-4-androstene-11,17-dione
Adrenosterone	Ad	4-androstene-3,11,17-trione
Synthetic androgens	1	
17α-Methyltestosterone	MT	4-androstene-17α-methyl-17β-ol-3-one
17α-Methyldihydrotestosterone	MDHT	5α-androstan-17α-methyl-17β-ol-3-one
17α-Ethynyltestosterone	4-androstene-17α-ethynyl-17β-ol-3-one	
Progestogens (C_{21})	1	
Progesterone	P_4	4-pregnene-3,20-dione
Pregnenolone	P_5	3β-hydroxy-5-pregnen-20-one
17-Hydroxyprogesterone	$17P_4$	17-hydroxy-4-pregnene-3,20-dione
17-Hydroxypregnenolone	$17P_5$	3β,17-dihydroxy-5-pregnen-20-one
17,20β-Dihydroxyprogesterone	DHP	17,20β-dihydroxy-4-pregnen-3-one
Corticosteroids (C_{21})	11β,17,21-trihydroxy-4-pregnene-3,20-dione	
Cortisol		17,21-dihydroxy-4-pregnene-3,11,20-trione
Cortisone		5-cholesten-3β-ol
Cholesterol (C_{27})	1	

chorionic gonadotropin (hCG) induced in all cases a significant percentage of female-to-male sex inversions. The fact that one single injection can initiate a complete sex inversion in this species is in accordance with the hypothesis that a short rise in gonadotropic secretion will trigger sex inversion without the need for high sustained secretions [55, 56]. In the same species, sex inversion has been shown to be associated with a two- to threefold increase in the number of immunoreactive GnRH cells in the preoptic area of the brain [57], and injection of GnRH-analogue (GnRH-A) in combination with domperidone (DOM, a dopamine receptor antagonist) results in 92% of inversion after 6 weeks of treatment [58]. In the ricefield eel, treatment with GnRH-A does not promote sex inversion regardless of the female stage [53, 54], although a different experiment in the same species has revealed that long-term treatments with GnRH-A, with or without DOM, induced sex inversion of females [59]. At an even higher level of brain control, neuropeptide Y (NPY) treatment of female bluehead wrasse leads to 100% of animals undergoing sex inversion [60]. This action of NPY is thought to act on the hypothalamo-pituitary axis through the stimulation of GTH secretions in much the same way NPY has been shown to stimulate GTH II release from the pituitary gland in adult fish of other species [61]. In the closely related species *Thalassoma duperrey*, the involvement of catecholamines in the process of sex inversion has been demonstrated, and they are thought to trigger changes along the hypothalamo-pituitary-gonadal axis [62]. The possibility of steroid feedback in the brain has also been investigated. For instance, in the bluehead wrasse, 11-ketotestosterone (11KT) implant treatments of primary phase animals result in a complete transition to terminal phase coloration and concomitantly in an increase in the number of GnRH cells in the forebrain preoptic area [63, 64]. This increase mimics the twofold increase of these cells detected between primary female phases and terminal male phases in this species [57].

In protandrous fish, the results are less clear-cut as treatments involving either GTHs or GnRH-A have not been demonstrated to be particularly effective in inducing sex inversion. In the seabream *Sparus aurata*, however, large differences are found between the expression of the GTH I subunit β (βGTH I) and βGTH II in males and females, βGTH I being higher in males and βGTH II higher in females [65]. Long-term treatment with GnRH-A was shown to induce the development of the ovarian part of the ovotestis [66]. Despite numerous attempts, treatments with GnRH-A in another sparidae, the black porgy, *Acanthopagrus schlegeli*, have failed to induce sex inversion [67–69]. In this species, however, concentrations of GTH II, as well as the expression of the βGTH II gene, have been shown to be higher in the pituitary glands of females undergoing natural sex inversion [69]. Furthermore, estradiol-17β (E_2) treatments have also been shown to efficiently induce sex inversion while at the same time stimulating gene expression and secretion of GTH II, but not GTH I [70]. All these studies suggest that GTH II may play an important role in triggering sex inversion in protandrous species, and this could also be the case in some protogynous species as is suggested in the ricefield eel [51, 52].

In the protandrous hermaphrodite anemonefish *Amphiprion melanopus*, the number of GnRH-producing cells in the preoptic area (POA) but not in the terminal nerve (TN) differs between the different sexual phases with a significantly higher number during the male phase. This suggests that this POA cell population plays a central role in the initiation of male-to-female sex inversions in this species and acts as a neuronal system mediating the social control of sex and gonadogenesis [71].

Involvement of steroid hormones in gonochoristic species

Parabiose experiment. In the medaka, trunks of newly hatched fry were transplanted into the anterior eye chamber of adult animals of both sexes. When transferred into a male host, the gonads of a genetic female graft develop into an abnormal gonad structure containing spermatogenetic cells. When transferred into a female host, the gonads of a genetic male graft still develop into testes [72]. It was concluded that differentiation of male germ cells requires 'male sex hormones' whereas differentiation of female germ cells is not induced by physiological levels of 'female sex hormones'. Among these putative sex hormones, steroids have received the most attention.

Involvement of steroid hormones in gonochoristic species. Present knowledge concerning the role of steroid hormones in the process of sex differentiation has been mainly acquired through indirect techniques such as the use of treatments with steroid hormones, steroid enzyme inhibitors or steroid receptor antagonists. The data obtained from these experiments are only exploitable when an effect on the resulting sex ratio is found, as an absence of effect can be due to inadequate timing, dosage or mode of administration of the compounds. Moreover, most of the inhibitors or antagonist molecules used in fish have only been proven to be active in mammals. Measuring steroid hormone levels during gonadal differentiation or describing the steroid enzyme potentialities in the differentiated gonads also provides important information. In addition, steroid-producing cells have been identified during the process of sex differentiation in a few species (reviewed in [8]).

Steroid treatments. Yamamoto postulated in 1969 that steroids were the natural 'sex inducers', oestrogens being the 'gynoinducers' and androgens being the 'androinducers' [1]. From that moment on, a vast number of experiments were conducted dealing with steroid treatments in fish. The aim of most of these treatments has been to control the sex phenotype in fish species of commercial interest [12, 17]. Thus, only studies which provide some physiological insight as to the regulation of sex differentiation by steroid hormones will be examined here.

First of all, it should be specified that the gonadal sex phenotype in gonochoristic fish can only be manipulated around the time of the sex differentiation period. However, in the adult goldfish *Carassius auratus*, 11KT implant treatments in incompletely ovariectomized females can lead to the development of testicular tissue, suggesting that germ cells can still retain a certain level of bipotentiality [73]. This bipotency of germ cells has also been suggested by in vitro experiments in the same species [74], and in vivo in the medaka [75].

Apart from the numerous treatments that are known classically to be effective in inducing feminization (using oestrogens) or masculinization (using androgens), there exist a number of paradoxical results on the feminizing effects of androgens in fish [76–80]. In a genotypic all-female population of Chinook salmon *Oncorhynchus tshawytscha*, balneation treatments were performed with either the synthetic, aromatizable methyltestosterone (MT) or the synthetic, nonaromatizable androgens methyldehydrotestosterone (MDHT), and the relative masculinizing potencies were MDHT > MT. Thus, it seems that the aromatization of androgens decreases their relative masculinizing potencies [81]. However, in the channel catfish *Ictalurus punctatust* and the blue catfish *Ictalurus furcatus*, paradoxical feminizations have also been observed following treatments with non aromatizable androgens [82, 83], and these types of puzzling feminization effects remain to be explained. Certain oestrogenic effects of androgens in differentiated fish, however, have been suggested to be modulated directly by oestrogen receptors [84].

In the Coho salmon, two well-differentiated periods of steroid sensitivity have been demonstrated with a maximum efficiency of feminization with E_2 balneation treatment carried out 1 day after hatching and a maximum efficiency of masculinization with MT 1 week later [85]. Although the effects of steroid treatments on masculinization or feminization have been well documented, very little data exist concerning the action mechanisms of these steroids. For instance, aromatization is thought to decrease the masculinization efficiency of some aromatizable androgens [78, 81], but whether this is due to a decrease in androgen concentrations or an increase in oestrogen concentrations is unknown. The physiological effects of steroid treatments on the differentiation of gonads has received very little attention. Selective incorporation and accumulation of radiolabelled steroids have been demonstrated in the gonads of medaka [86–88]. In rainbow trout, E_2 or MT treatments performed on all-male and all-female populations at the onset of the first feeding inhibited the in vitro production of steroids in both sexes, and did not induce the gonadal steroid secretion pattern of the opposite sex [48]. In the common carp, treatments with E_2 failed to feminize XY males, but produced intersexed animals on $XX_{(mas/mas)}$ males (animals homozygous for a recessive mutation in a gene called mas for mas-culinization). Together with the fact that these $XX_{(mas/mas)}$ males (but not normal XY males) produced oestrogens, it seems that increasing endogenous oestrogen levels drive differentiation slightly towards female differentiation but without totally overriding the masculinizing effects of endogenous 11-oxygenated androgens [89].

Apart from these common treatments, a few studies have shown some effects of corticosteroids [90] or progestins [91] on sex differentiation. For corticosteroids this effect could be explained by a conversion into 11-oxygenated androgens [90].

In vivo treatments with steroid enzyme inhibitors. In the salmon *O. tschawytscha*, balneation treatment of all-female populations with a nonsteroidal aromatase inhibitor (AI) resulted in induced masculinization and increased masculinization induced by a low dosage of MT. Thus AI, by reducing aromatization of MT, greatly enhances the masculinizing effect of an aromatizable androgen [92]. In rainbow trout and in tilapia *Oreochromis niloticus*, treatments with the steroidal aromatase inhibitor 1,4,6-androstatriene-3-17-dione (ATD) resulted in masculinization of all-female populations [93–94]. High-dosage treatments of rainbow trout with cyanoketone, a 3β-hydroxysteroid dehydrogenase (3βHSD) inhibitor, resulted in a significant increase of males. Surprisingly, this treatment also resulted in an important increase in the 3βHSD activity in the interrenal tissue, but not in the gonads of treated fish. This could result from indirect stimulation of steroidogenesis in the interrenal tissues producing 11β-hydroxyandrostenedione (11βOHΔ_4), which is able to masculinize the gonads [49]. However, treatment with metopyrone, an 11β-hydroxylase (11βH) inhibitor, failed to induce feminization, but inhibition of 11βH was found to be ineffective, at least in the gonads [95].

In vivo treatments with steroid receptor antagonists. Nearly all attempts to masculinize or feminize fish using steroid receptor antagonists have failed [93–96]. Only one unpublished report [cited in [96]] mentions that the androgen receptor antagonist cyproterone acetate induces feminization in the medaka. For oestrogen-receptor antagonism, one experiment reports a masculinizing effect of tamoxifen in a hybrid population of tilapia *O. niloticus* × *O. aureus* [97].

In vitro steroid metabolism. Some steroidogenic potentialities of eggs or very young embryos have been shown in different species of salmonids [98, 99]. These pathways are thought to be mainly deactivations of active steroids of maternal origin [99]. Few studies have investigated the steroidogenic potentialities of the differentiating gonads during the period encompassing sex differentiation. In the tilapia *O. niloticus*, aromatase activity was specific for ovaries shortly after differentiation, whereas 11βH and 11β-hydroxysteroid dehydrogenase (11βHSD) activities were specific for testes [95, 100]. The same pattern has been observed during gonadal differentiation in the rainbow trout (authors unpublished results; see fig. 1). In the same species, the undifferentiated gonads have been shown to possess 3βHSD and 17-hydroxylase (17H) activities. Shortly after gonadal differentiation, testes are also able to synthesize 11βOHΔ_4 and adrenosterone (Ad). In females, there are no 11-oxygenated androgen synthesis potentialities, and oestrogen synthesis potentiality is only acquired much later following gonadal differentiation [101, 102]. In larvae of the catfish *Clarias gariepinus*, 11-oxygenated androgens were also specific for testis differentiation [103]. In the common carp, the major metabolites detected in XY males are 11-oxygenated androgens, with nearly no oestrogens, whereas in $XX_{(mas/+)}$ females, oestrogens are the major metabolites with no 11-oxygenated androgens produced at all. In $XX_{(mas/mas)}$ males, both oestrogens and 11-oxygenated androgens are produced, and animals differentiate into the male phenotype. In conclusion, it would appear that the precocious synthesis of 11-oxygenated androgens in $XX_{(mas/mas)}$ animals directs male testicular differentiation [89].

Steroid assays. Because of the very small size of differentiating gonads in fish, many studies carried out to measure steroids during sex differentiation have been performed on eggs, embryos or whole-body extracts. Most of these studies have come to the conclusion that steroids, some of them probably of maternal origin, can be detected and metabolized very early [98, 99, 104]. Interpretation of these experiments is difficult since extragonadal steroid production sites exist in fish [105, 106], and differences between sexes have rarely been found [107]. However, using all-male and all-female populations of rainbow trout, it has been demonstrated

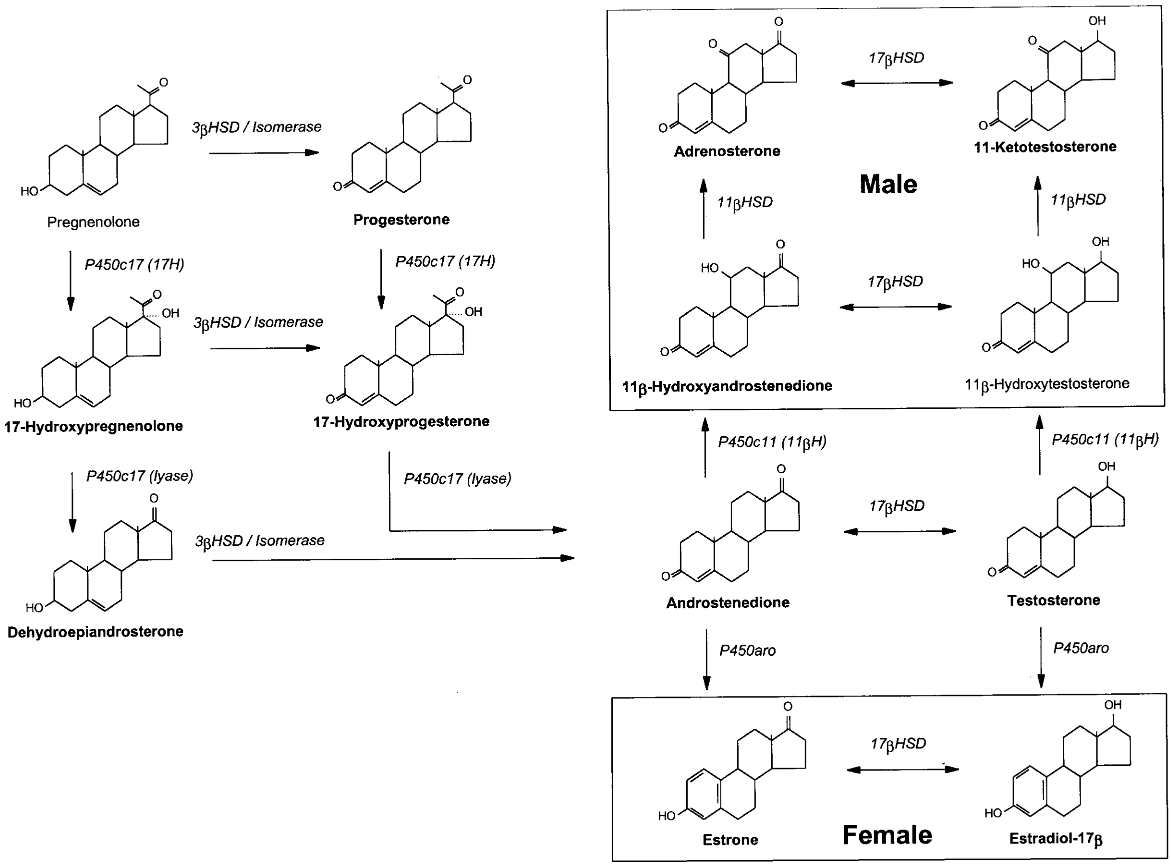

Figure 1. Schematic representation of some gonadal steroidogenetic pathways in fish and identified steroids (in bold type) following in vitro steroid metabolism with pregnenolone and androstenedione precursors in rainbow trout and/or tilapia differentiating gonads (authors' published results). Enzyme activities are $P450_{aro}$, aromatase; P450c11 (11βH), 11β-hydroxylase; P450c17 (17H), 17-hydroxylase; P450c17 (lyase), 17,20-lyase; HSD, hydroxysteroid dehydrogenase.

that higher levels of Δ_4 and T are produced in the testis, 2 weeks before histological gonadal differentiation. Gonadal secretion of E_2 is higher in female gonads but only after histological gonadal differentiation [48]. In *O. niloticus*, T levels are only detectable in the gonads and serum after testicular differentiation [108]. Also, a transient peak of T and 11KT has been detected in gonads at 22 dpf and a peak of E_2 during ovarian differentiation [109].

Steroid enzyme immunodetection and gene expression. In the eel *Anguilla anguilla*, cytochrome side-chain cleavage ($P450_{scc}$) was immunocytochemically detected in gonads before sex differentiation using a rat antibody [110]. During the process of sex differentiation in *O. niloticus*, two patterns of immunoreactivity were detected in gonads, one with a positive staining for all antibodies tested ($P450_{scc}$, 3βHSD, cytochrome 17-hydroxylase/17,20 lyase = $P450_{c17}$, aromatase = $P450_{aro}$) and one with no staining at all. These positive staining reactions became detectable a few days before morphological sex differentiation and increased thereafter. Based on the positive staining for $P450_{aro}$, these gonads were assumed to be female. Weak positive reactions were detected at 30 dpf in presumed testis, but $P450_{aro}$ immunostaining was never detected [18]. Also in the rainbow trout, expression of the $P450_{aro}$ gene is specifically detected and highly expressed only in female gonads 2 weeks before the first sign of histological differentiation [93] (fig. 2). Conversely, $P450_{c11}$ gene (11β-hydroxylase) is highly overexpressed in male gonads as early as specific $P450_{aro}$ expression is found in female gonads (authors unpublished data; fig. 2).

Steroid receptivity. Although steroid enzyme potentialities have been studied in some fish, the problem concerning the receptivity of the differentiating gonads has only occasionally been addressed. In rainbow trout, oestrogen receptor genes are expressed early in both male and female gonads before and during sex differentiation, showing that gonadal receptivity for oestrogen may be acquired by both sexes [93] (fig. 2).

Involvement of other factors. Genes previously found to be important in the process of gonadal sex differentiation in mammals have also been found in fish such as the Wilms's tumour predisposition gene (WT1), which has been cloned in the pufferfish *Fugu rubripes* [111] and in the zebrafish *Brachydanio rerio* [112]. The steroidogenic factor 1 (SF1), or SF1 homologues belonging to the FTZ-F1 family, have also been found both in the zebrafish [113, 114] and rainbow trout [115].

Finally, a Sox9 gene [31] and some SRY-related sequences [30, 32, 33] have been described in rainbow trout. None of these genes has been studied with respect to gonadal differentiation events, however.

Finally, two sequence homologues to *zona pellucida* proteins ZP2 and ZP3 were shown to be differentially expressed in the medaka gonads starting from 5 days post-hatching [116].

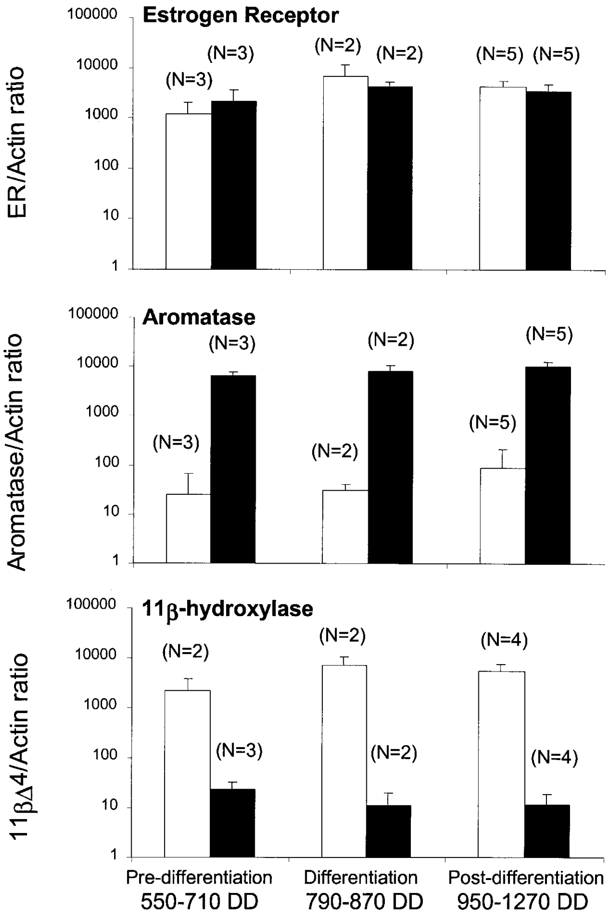

Figure 2. Semiquantification by RT-PCR (reverse-transcription polymerase-chain reaction) of expression of some genes in gonads of rainbow trout before (predifferentiation), during (differentiation, i.e. first oocyte meiosis in females) and after (postdifferentiation) histological gonadal differentiation (authors' unpublished results). Males are represented by open bars and females by black solid bars. Data are represented as ratio of the gene of interest over β-actin gene and expressed using a logarithmic scale. DD, degree × days. Details on the semiquantitative technique will be published elsewhere.

Involvement of steroid hormones in sex inversion of hermaphroditic species

Steroid hormones and sex inversion have been quite extensively studied. The question still remains, however, whether sex inversion and sex differentiation can be considered to be physiologically related processes [8]. Another problem in studying the physiology of sex inversion is the difficulty in linking steroid levels or steroid metabolism and the sex inversion process, because in many cases these descriptions are the result of two different physiological events, i.e. reproduction and sex inversion [116]. In this regard, most of the studies performed on hermaphrodites have compared only male and female stages, and these are more likely to reflect differences between male and female reproductive characteristics than changes linked to the regulation of sex inversion.

Steroid treatments. In protogynous species, androgen treatments have generally been shown to be effective in inducing female-to-male inversions. In two labridae species, *Thalassoma bifasciatum* and *Halichoeres bivittatus*, sex inversion was obtained by a single injection of testosterone (T) [118]. In another labridae, *Sparisoma viride*, one single injection of 11KT was observed to induce sex inversion [119]. However, in the ricefield eel neither T, MT nor 11KT was effective [51]. Treatments with MT have been shown to be effective in different species of grouper [119–126]. In some of these studies, however, the sperm obtained from these steroid-treated males had no fertilizing ability [120], or males reversed back to a female status the following year [122, 126]. In a study on *Epinephelus tauvina*, it has been suggested [121] that the action of MT can be mediated by either a direct action on gonads or by feedback via the CNS and subsequent stimulation of gonadotropin secretion and/or release. Regarding the accumulation of labelled T by the gonads and CNS following hormonal injection in *T. bifasciatum* [127], both hypotheses remain possible. In protandrous species, all oestrogen treatments have been carried out in the family Sparidae. In the black porgy *Acanthopagrus schlegeli*, treatment with E_2 before the reproduction period induced 100% sex inversion of 2-year-old males [128]. In the seabream, E_2 also enhances sex inversion [129, 130]. In the black porgy and in *Sparidentex hasta*, E_2 treatment also feminized immature fish [131–133].

In vitro steroid metabolism. Steroid metabolism has been well studied in gonads of hermaphroditic fish, and the enzymatic pathways detected are generally similar to those found in gonochoristic species with an important variation from one species to the next [134, 135]. These studies have been carried out on many different species and, as a result, no general statement can be made as to the possible correlation between certain enzymatic activities and the sex inversion process [134]. Apart from many comparisons between male and female status, very few studies have addressed the problem of changes in steroidogenesis during the sex inversion process. In the tropical protandrous seabass *Lates calcarifer*, a very high production level of an oestrogen-like compound has been detected in the mid-transitional stages [136]. In the seabream, sex inversion induced by E_2 leads to a concomitant increase in E_2 and a decrease in 11KT production in vitro [130]. The protogynous ricefield eel has been the subject of extensive studies related to steroid metabolism. During natural sex inversion there is a clear increase in the androgen-to-oestrogen ratio [137, 138]. A transition occurs from a femalelike pattern characterized by the production of 5α-reduced compounds to a malelike pattern characterized by the production of 11KT [54]. Following masculinizing treatment with ovine LH, E_2 decreases, and $11\beta OH\Delta_4$, Ad and 11KT increase [54].

Steroid assays. In the protandrous sparidae *Rhabdosargus sarba*, steroid plasmatic levels in transitional phases are roughly similar to those found in males [135, 139]. In the protandrous anemonefish *Amphiprion melanopus*, the E_2/11KT ratio exhibits a clear increase dunng sex inversion [140]. In *Sparidentex hasta*, a peak in serum concentrations of E_2 is observed in males. In fish that remained male, E_2 levels fell and 11-oxygenated androgens increased, while fish that underwent sex inversion exhibited rising E_2 levels and low 11-oxygenated androgen concentrations. These results suggest that sex inversion occurs when threshold levels of oestrogens are secreted from the ovarian part of the ovotestis [133]. In the tropical seabass, gonadal E_2 is the only steroid detected in transitional gonads even during degeneration of the testicular tissue [136, 141], and there is a clear decrease in the plasma androgen/oestrogen ratio during sex inversion [136]. In the protandrous black porgy, only E_2 displayed different levels between males that stayed males and males that underwent sex inversion. In these fish, there was a transient peak of E_2 during the early prespawning season [131]. Following feminizing treatment using E_2, significantly higher E_2 plasma levels were observed prior to the reproductive season [142]. In the protogynous grouper *Epinephelus morio*, the E_2-to-androgen ratio has been shown to decline dramatically during sex inversion [143]. In *Sparisoma viride*, fish undergoing sex inversion are characterized by elevated plasma levels of 11KT, unchanged levels of T and low levels of E_2 [119]. Treatment with MT in the grouper *E. fario* increased serum T concentrations [120]. In the ricefield eel, plasmatic levels of Δ_4 have been shown to increase in the early and mid-transitional phases and then to drop dramatically in late transitional and male phase. After a slight increase in E_2 seen in the early transitional phase, E_2 decreases to very low levels in the mid- and late transitional phases. 11-Oxygenated androgens levels are very low to nearly undetectable regardless of the sex considered. These steroids are thus considered to have no functional significance in the process of sex inversion in this species even if they are produced in vitro [135, 117]. During natural or GnRH-A-induced sex inversion in the ricefield eel there is an increase in plasma levels of T and a decrease in E_2 [52, 59].

Steroid enzyme immunodetection and gene expression. In *T. duperrey*, expression of the $P450_{aro}$ gene is detected only in female gonads, and aromatase messenger RNA (mRNA) is no longer detected even in the earliest stages of sex inversion. This has led to the suggestion that downregulation of the $P450_{aro}$ gene is necessary to enable male differentiation [144]. In addition, $P450_{scc}$ immunoreactive cells have been detected among the theca cells of the female phase, and this localization shifts to the interstitium in sex-inverting animals. Furthermore, the number of these $P450_{scc}$ immunoreactive cells seems to increase in the gonad throughout sex inversion. In the male phase, these cells can be found in small clusters interspersed among spermatogenic lobules after sex inversion [145].

Other gonadal factors involved in sex inversion. In *Halichoeres poecilopterus*, a specific protein named p26 is found in the transitional gonad. This protein shares high homologies with ubiquitin C-terminal hydroxylase, an enzyme that cleaves ubiquitin and is associated with nuclear proteins and a transcriptional repressor. Thus, p26 may be involved in the regulation of gene expression during sex inversion [146].

Influence of environmental factors on sex differentiation

Environmental factors influencing gonadal sex differentiation and/or sex inversion

Hermaphrodites. The various factors thought to induce or modulate sex inversion in hermaphroditic fish have been reviewed elsewhere [4, 7, 147]. Very few studies, however, have been undertaken concerning the influence of environmental factors on primary gonadal sex differentiation in hermaphroditic fish.

Temperature. The unique study examining the effect of temperature on hermaphroditic teleosts was carried out on *Rivulus marmoratus* [148–152]. In this normally synchronous hermaphrodite, the spontaneous occurrence of primary males (through direct sex differentiation) was determined to be less than 5%. Low-temperature treatments (at or below 20 °C) increased this proportion to over 35%, whereas high temperatures induced sex inversions and, thus, the production of secondary males [148, 149, 151]. Mortality could not account for these differences, suggesting that temperature directly affects sex differentiation (primary male production) and/or sex inversion (from synchronous hermaphrodites to secondary males) in *R. marmoratus* [150].

Photoperiod and light intensity. Light intensity and photoperiod do not appear to have any direct effect on sex differentiation or sex inversion in *R. marmoratus*. Sex inversions can be favoured, however, when short-day lengths (12 h or less) are applied following an early rearing treatment at high temperatures [150]. Conversely, the response to photoperiod treatments is delayed or suppressed following low to moderate rearing temperatures during early growth periods.

Social factors. Since the pioneer studies on *Anthias squamipinnis* [153], the existence of a behavioural control of sex inversion has been well established in about 12 hermaphroditic species with developed reproductive and social systems [154–162]. As a general rule, a gradual or sudden perturbation of social interactions between individuals of a given group will result in a complete reversal of the reproductive orientation (e.g. gonadal, enzymatic, antigenic, behavioural) of one or several individuals [163–165]. Death or removal of a male within a protogynous group, or a female within a protandrous group, induces sex inversion of the largest individual of the opposite sex [153, 154, 156, 166]. Some of these social factors appear to be of major importance for the initiation of sex inversion in a number of species [153, 166–168]. The main hypotheses proposed to explain the proximate causes of socially controlled sex inversion are as follows:

1) Presence/absence of a sex type:

Presence or absence of a male within a social group constitutes the main factor governing the sex inversion process in protogynous coral fish species. Removal of the male from a mixed group of *Anthias squamipinnis* will result in the complete sex inversion of one female. Similar results can be reproduced by the removal of this new male [160]. Sex inversion of several females can also be obtained within an experimental all-female group of *Gobiosoma multifasciatum* and *Coryphopterus personatus* [169]. Thus, the absence of a male in a social group seems to induce sex inversions in these protogynous hermaphroditic species. However, the largest individual within an all-female group of *A. squamipinnis* will dominate and be considered as a male by the other females. Contrary to removal experiments, this individual will never change into a functional male nor acquire typical, complete, male behaviour [170]. Moreover, in such monosex groups, a minimum number of females is needed for sex inversion to occur [160, 171, 172]. Despite the presence of other males within the group, the removal of one male from a multiple-male social group of *A. squamipinnis* also induces the female sex inversion of a single female. Thus, absence of a male is not the only factor regulating sex inversion in protogynous species [160, 171, 173, 174]. Finally, the simultaneous removal of *n* males from a multiple-male social group of protogynous species results in successive sex inversion of *n* females with a mean 2-day delay between the onset of the successive sex inversions [167]. Thus, the sex inversions occurring within a social group will influence the sex inversion of a given individual.

2) Hierarchy and aggressiveness:

Hypothesis concerning inhibition release. In the protogynous *Labroides dimidiatus*, sex inversion is inhibited by the presence of an aggressive, dominant male within its 'harem' of females. Such inhibition is released by the death or removal of this male, and thereafter the largest dominant and aggressive female undergoes a sex inversion [167]. Similar observations have been reported in the protandrous *Amphiprion alkalopisos* and *A. bicinctus*, in which sex inversions are inhibited by females through an aggressive dominance over males [154]. Thus, sex inversion is influenced by an alteration of the social status of individuals within a group (induced by male or female removal) rather than by the absence of a male, as suggested in other field observations on *Thalassoma bifasciatum*, *Paragobiodon* sp., *Amphiprion melanopus*, 6 other species of *Amphiprion*, *Centropyge interruptus* and *C. resplendens* [156, 159, 164, 173, 175–177]. Six different profiles of behaviour (rushes, lateral dorsal displays, bent approaches, mouth to mouth, nose bump/crosses and U-swims) received or given by a member of a social group have been described in *A. squamipinnis* [178]. These profiles will be affected by the removal of males, and the degree to which they are altered will depend on the dominance rank of females [174, 178]. The most dominant and aggressive female will acquire a typical male profile of behaviours received and given and be immediately considered as a male by the other females, and this from the onset of its sex inversion. Within an all-female group of *A. squamipinnis*, however, the mere presence of a similarly dominating female is not sufficient to induce its sex inversion in the absence of a male removal [178]. Each member seems to integrate a relative proportion of the behaviours given and received from all the other individuals: the threshold value for a sex inversion of the dominant female will only be attained following a male removal, and in the presence of a minimum number of other females [160, 174, 178]. Such a hypothesis could explain the absence of (or the long period required to initiate) sex inversion in experimental all-female groups. In two species of the anemonefish *Amphiprion* sp., death or removal of the dominant female induces sex inversion in its mature mate. The mature male condition is then assumed by the dominant individual from the satellite group composed of unrelated immature members [154].

Hypothesis of necessary priming. Due to the existence of numerous all-female groups in a number of protogynous species [161, 172, 179], the hypothesis describing sex inversion as being regulated not only by the release of an inhibition but also by an active stimulation prior to the removal of the aggressive dominant member has been developed [168]. If sex inversions were only induced by the release of an aggressive inhibition, similar timing would be observed for the onset of such changes within all-female or mixed groups. Sex inversions, however, require a longer time period in monosex groups [147, 180]. Moreover, in species such as *A. squamipinnis*, the removal of a male may be necessary to prime the sex inversion of the dominant female. Male removal induces a dramatic alteration in a set of discrete behaviours which will actively stimulate the dominant female [160]. In the anemonefish *Amphiprion* sp. [154] as well as in *T. duperrey* [161], experimental female pairs resulted only in very aggressive interactions without the occurrence of any sex inversion.

Sex ratio and critical number of males and females. The onset of sex inversion may also be modulated by either the sex ratios [160, 181, 182], and/or a minimal number of individuals of each sex [168]. In *T. duperrey* and *A. bicinctus*, at least two males and two females are needed for a sex inversion [154, 161, 183]. In the Sudanese Red Sea groups of *A. squamipinnis* and in *Labroides dimidiatus*, sex inversion can be induced by reaching a critical sex ratio threshold [160, 182]. Within a given species, however, the existence of all-female groups does not fit well with such a model. In addition, a minimal number of females (two to four) in protogynous species or of males (at least two) in protandrous species would appear to be necessary within a social group for sex inversion to occur after removal of the member of the opposite sex [165, 167, 171, 182]. This minimal number of potential breeders may be due to the existence of a relationship between the rate of sex inversion, fry recruitment rate, and male and female mortality rates [182]. This last hypothesis of a critical sex ratio would help explain some of the sex inversion rates observed under very specific culture conditions in the grouper *E. microdon*, following the accidental skewing of sex ratios. In these species, individual female-to-male and/or even male-to-female inversions take place to reach a balanced (1:1) sex ratio (reviewed in [136, 164, 184]). In the protandrous tropical sea bass *Lates calcarifer*, a 45% sex inversion rate was recorded within a male population of a similar age. In this species, it is thought that a low proportion of females (26.5%) or a high proportion of males (73.5%) and/or experimental groups limited in size (due to culture conditions) may influence the sex inversion rate through an intensification of behavioural signals [141, 185].

Critical density. Sex inversion is initiated when a critical low density is reached in populations of *Gonostoma bathyphylum* [186], whereas high densities favour precocious sex inversions in *Coris julis* [187].

Nature of the social stimuli. The exact nature of the social stimuli suspected to be involved in sex inversion initiation remains to be determined [188]; the relative importance of visual cues has been investigated in very few species. Visual cues are considered to be crucial in *T. duperrey*. Indeed, studies using barriers which allow water flow but obstruct vision revealed that sex inversion is controlled socially and initiated by visual stimuli [168, 189, 190]. In *A. squamipinnis*, isolation of a male in an opaque or transparent cylinder allowing chemical or sound transmissions was functionally similar to complete male removal, and sex inversion occurred in equal proportions for both of these treatment groups [191]. In the same species, however, sex inversions can be delayed in a monosex group if the females are able to see a male behind a glass window [153, 192]. Loss of male-female interactions seems to be the most important factor for sex inversion. The cues indicating the presence of other members of the same or opposite sex may be visual [168] or chemical (Coel and Shapiro, unpublished data, cited in [165]). However, behavioural sex inversions were still observed in the absence of gonads in a coral reef fish *T. bifasciatum* [193]. Tactile cues are not needed for sex inversion to occur in *T. duperrey* [189].

Interactions between genotype and environmental factors. In *Rivulus marmoratus*, sensitivity to temperature is highly variable between different laboratory lines. The rate of spontaneous primary male production and sex inversion precocity (production of secondary males) also differs greatly between the different lines [152]. Similarly, the number of short-day seasons required for the induction of a sex inversion has been found to be genotype-specific in this same species.

Gonochoristic species. In gonochoristic species, sex differentiation can be influenced by exogenous factors. Most studies have focussed on the effect of exogenous steroids on sex differentiation. Although the influence of social factors in the regulation of sex inversions in certain hermaphroditic species has been well described, the presentation of indisputable evidence concerning the effect of environmental factors on sex differentiation in gonochoristic fish is more recent. As a result, very few environmental factors have been studied in only a limited number of species. Moreover, it is important to note that a number of studies concerned with the effect of environmental factors on sex differentiation have used, deliberately or not, inbred lines of fish species. This is particularly true for those works using species from the poecilids family as laboratory models. The relative importance of such inbreeding on the sensitivity to environmental factors remains to be determined [11]. In that regard, inbreeding has been shown to induce spontaneous XX male production (fixation of rare masculinizing recessive alleles) at least in the common carp [194, 195] and the rainbow trout (E. Quillet, unpublished data, cited in [9]). In gonochoristic fish species, as in other vertebrates displaying environmental sex determination (ESD), the main environmental determinant of sex may be the prevailing temperature during early development [196, 197]. This also happens to be the factor which has received the greatest attention. Most of the studies on temperature effects have focused on reptiles and amphibians: temperature effects, also called temperature sex determination or TSD [196, 198] have been demonstrated in a wide variety of crocodiles, turtles, lizards and certain species of frogs and salamanders [10, 197]. In amphibians, these temperature effects may only be artefacts due to the abnormally high rearing temperatures used, and thus under natural conditions, sex could depend exclusively on genetic factors [10]. In fish, the putative effects of other factors such as pH, salinity, photoperiod or social interactions have received much less attention.

Effect of pH. The strong influence of pH, either alone or through interactions with temperature, has been reported in a number of species. In *Xiphophorus helleri*, the development of male monosex populations (100%) or nearly female monosex populations (< 2% males) is obtained at an acid pH (6.2) or at a slightly basic value (7.8), respectively [199]. Similar results have been described in another poecilid, *Poecilia melanogaster* [200], and in 7 out of 37 species of *Apistogramma* (cichlids) under study: male proportions are inversely proportional to pH [199, 200]. In *A. caetei*, for example, balanced populations are observed at an acid pH (53–60% males/pH 4.5–5.5), whereas under more neutral conditions, almost exclusively female populations are produced (4% males/pH 6.5).

Density. In the paradise fish *Macropodus opercularis* [201], individual isolation favours testicular differentiation (89% males), whereas grouping individuals induces a female differentiation which is proportional to the density (25% at the lowest density, 66% females at the highest density).

Relative size of juveniles. In the Midas cichlid, *Cichlasoma citrinellum*, stable size ranks are reported within a cohort from the juvenile stage to sexual maturity. Within a cohort of siblings, the larger individuals differentiate into males, whereas smaller members undergo female differentiation. Males are always larger than females and have the primary role of defending the breeding territory. Finally, females prefer large males. The experimental alteration of size distribution (grading according to size) within a group results in a slight deviation in favour of females in both initially lower- and upper-size groups; assuming that sex is exclusively determined by genetic factors, both male (fish above the median size) and female (below the median size) groups should be obtained. Therefore, sex differentiation can be influenced by relative size [202].

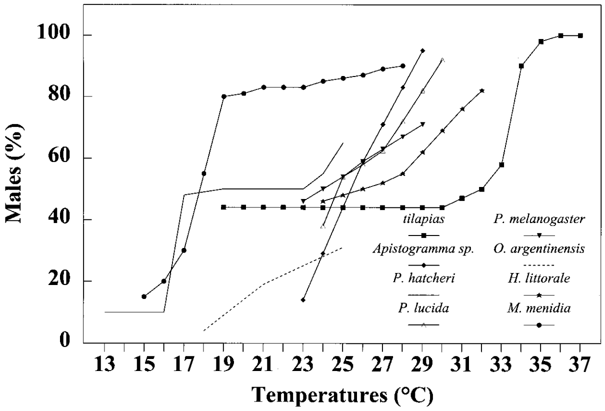

Figure 3. Masculinization by high temperatures and/or feminization by low temperatures in seven fish species with temperature-dependent sex determination (schematic representation of the extreme data from [200, 203–216]).

Effect of temperature on sex differentiation: different models of thermosensitivity

In gonochoristic fish, although the thermosensitivity of gonadal sex differentiation is being discovered in an increasing number of species (as more research is carried out on this topic), it is probably premature to attempt to define TSD patterns. In an effort to synthesize the main results, however, three main response types have been retained here:

1) In most of the species identified as thermosensitive, such as *Hoplosternum littorale* [203], *P. lucida* [204] and *P. melanogaster* [200], the proportion of males increases with temperature and/or female differentiation is favoured by low temperatures (fig. 3). In the tilapias *O. niloticus* [205–208], *O. aureus* [209] and the red tilapia (a four-way hybrid) from the Red Florida strain [210], low temperatures do not affect the sex ratio. Conversely, in the atherinids *Menidia*

menidia [211–213], *Patagonina hatcheri* [214–215], *Odonthestes bonariensis* [215, 216] and *O. argentinensis* [214], predominantly female populations are produced at low temperatures. Finally, it can be stated that, contrary to TSD patterns in reptile species [197], a complete change from monosex female to monosex male populations at extreme temperatures is never observed, with the exception of *O. bonariensis* [215, 216]. This last species differs from reptiles in that it lacks the abrupt threshold of TSD observed in the latter vertebrates [217].

2) In only two species, it is suggested that high temperatures may favour ovarian differentiation and/or low temperatures induce testicular differentiation (fig. 4). Indeed, monosex male populations of the sea bass *Dicentrarchus labrax* can be obtained at low temperatures (15 °C), whereas variable but low proportions of females (< 27% females) are produced under more typical rearing conditions (24–25 °C) [218]. A slight feminization (63% females) is induced by high temperatures (34 °C) in the catfish *Ictalurus punctatus* [219], whereas low and intermediate temperatures do not affect the typical balanced sex ratio (1:1). It should be noted, however, that these last two studies were carried out on a limited number of progenies.

3) Finally, only one species is currently known to produce monosex male populations at both high and low temperatures (U-shape curve): the hirame *Paralichthys olivaceus* [220]. At intermediate temperatures, the sex proportions observed fit well with a model of female homogamety (XX/XY): geneti-cally mixed populations (from pairs of XX females and XY males) generate balanced sex ratios whereas monosex female populations are sired by XX[3] males (fig. 5).

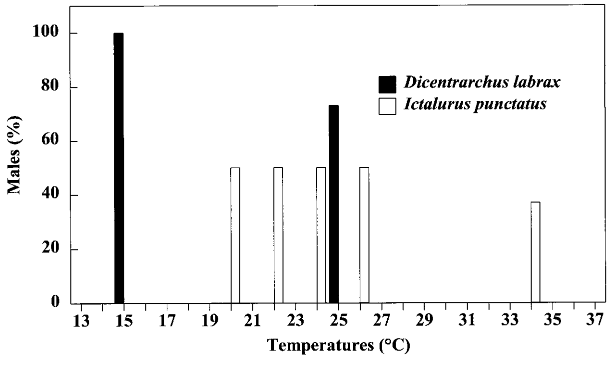

Figure 4. Feminization by high temperatures and/or masculinization by low temperatures in the European sea bass *Dicentrarchus labrax* and the catfish *Ictalurus punctatus*. Schematic representation of data from [218, 219].

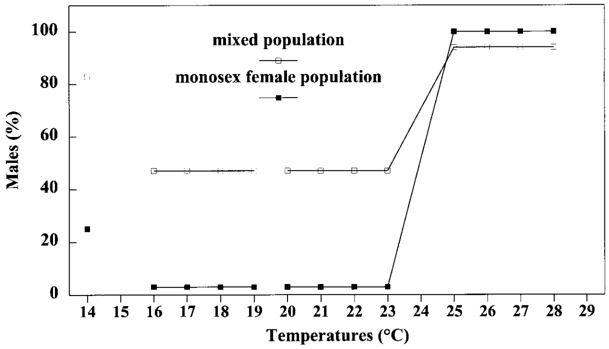

Figure 5. U-shape response to temperature in the Japanese flounder *Paralichthys olivaccus* in a mixed population and a genetically all-female population. Schematic representation of data from [220].

Thermosensitive period

In the tilapias [205, 206], some atherinids [215, 216, 221] and the sea bass [218], thermal treatments must begin before the onset of histological gonadal sex differentiation and must also at least partially overlap this critical period. In the above-mentioned species, as is true in reptiles, this period is very similar to the hormono-sensitive period, during which a hormonal treatment must be applied if such a treatment is to be efficient.

Genotype/temperature interactions

Species identified as thermosensitive can present all the possible genetic sex determination systems already described in fish [9, 24]. For example, in tilapias[3] [222] American catfish[4] [219] and hirame[5] [220] simple male or female homogamety[6] has been described, whereas more complex sex determinations (sex chromosomes cannot be identified even through indirect approaches) based on several major sex-determining factors are reported in the Atlantic silverside *M. menidia* [211].

In most thermosensitive fish species, interactions between environmental factors and genotype have been strongly suggested:

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[3] These males were produced through functional masculinization, and identified by their monosex female progenies: XX × XX → 100% female XX.

[4] XX/XY in *O. niloticus*, and ZZ/ZW in *O. aureus*.

[5] *Ictalurus punctatus* and *P. olivaceus* both exhibit a female homogamety XX/XY.

[6] A pair of homologous sexual chromosomes in the male (ZZ/ZW) or the female (XX/XY).

1) In *M. menidia*, a highly variable thermosensitivity has been reported both between and within populations. A sex-linked growth dimorphism has been described in favour of females. Females stemming from the very first, low-temperature reproductions will benefit from a longer growth season. As size preferentially favours the relative fecundity of females as compared with males, populations at different latitudes will compensate the thermal and seasonal differences by adjusting the magnitude of their response to temperature, and therefore adjust their relative thermosensitivity. As southern populations benefit from a long reproductive season (and subsequently a long growing season for the future generation), they present a high degree of thermosensitivity, and mostly female populations are produced. Conversely, northern populations are faced with a short reproductive season, and are not or only weakly thermosensitive [211, 213, 223]. Moreover, a high variability in thermosensitivity is also described within a given family [211].

2) In the tilapia *O. niloticus*, within a same strain, the most- and least-sensitive genetic female progenies (sired by XX males) will produce 98% and 5% males, respectively, at high masculinizing temperatures (36 °C) versus 100% and 96% females at control temperatures (27–28 °C is generally considered to be the optimal temperature for this species) [206]. Very similar results are observed using progenies sired from genetic XY males (fig. 3), and male proportions increase by 18–44% following masculinizing high-temperature treatments [205, 207]. Finally, parental effects are strongly suggested in certain tilapia species [210; J.-F. Baroiller and F. Clota, unpublished data]. A given female can successively mate with two different males, and produce highly sensitive (100% and 75% males at 36 and 27 °C, respectively) or nonsensitive (40% and 41% males at 36 and 27 °C, respectively) progenies. Similar results have been obtained with the progenies from two different females successively mated with the same male (61% and 82% males at 36 °C). Conversely, successive progenies from the same breeder pair always present similar thermosensitivities (58–65% males at 27 °C/80–83% at 36 °C).

3) Conversely, in another thermosensitive tilapia species, *O. aureus*, temperature/genotype interactions seem to be weak, and systematically all-male or almost monosex male progenies (95–100%) are produced following high-temperature treatment [209].

In *P. lucida*, only one out of two homozygous lines is thermosensitive. Segregation studies between the two lines suggest dominance of the thermosensitive trait [204]. In another poecilid, the guppy *P. reticulata*, an XX line seems to be more sensitive to environmental factors than a classic line due to fluctuating season-dependent sex ratios [224].

For species in which the effect of other environmental factors has been proven, similar genotype interactions have also been described: in the paradise fish *M. opercularis*, sex differentiation can be influenced by density, as has already been reported above [201]. After genetic selection of dominance in five successive generations, the positive line (where the dominance trait has been positively selected) presents a 77% proportion of males versus 52% in the control line.

Absence of temperature-dependent sex determination in fish

In light of the adaptive significance of TSD demonstrated in *M. menidia*, the hypothesis that other fish with similar life histories could also present TSD has been tested in two cyprinodontid fish, *Cyprinodon variegatus* and *Fundulus heteroclitus* [225]. Contrary to the expected hypothesis[7], low temperatures do not produce female offspring in *F. heteroclitus* nor do they generate male progenies in *C. variegatus* (43–58% females at 15, 18, 21 and 28 °C). Although the existence of TSD has not been demonstrated in the studied populations, the authors cautioned against drawing definitive conclusions for both species. Indeed, intraspecific variations in sex-determining mechanisms have been reported in several fish species [27, 29]. In the rainbow trout *Oncorhynchus mykiss*, the influence of temperature on sex differentiation has been investigated during the early developmental stages. High-temperature treatments (22–29 °C) applied soon after hatching and of various duration (10 min to 14 days) do not affect the sex ratio [226]. More recently, longer high-temperature (19 °C) treatments have been applied to genetic all-male and all-female populations during their entire hormonal sensitive period, and this without any effect on the respective sex ratios (present authors, unpublished data).

Genetic evidence of a functional sex inversion by temperature in gonochoristic fish

Temperature-induced sex masculinization has been demonstrated in sensitive tilapia progenies (79% males at 390 °C/46% males at 27 °C): after reaching sexual maturity, high-temperature-treated (HTT) and control males were individually progeny-tested. All-female (or almost monosex female) progenies were only detected in the HTT group, whereas progenies with balanced sex

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[7] In such a hypothesis, TSD can be considered partially responsible for the sexual size dimorphism in both *F. heteroclitus* (females are larger than males) and *C. variegatus* (males exceed females in size).

ratios were produced in both groups. The former all-female progenies were sired by XX males (genetic females functionally sex-inverted to males by high temperatures); conversely, balanced progenies resulted from mating using a genetic XY male [205, 207].

Discussion and conclusion

Information on the role of the CNS on gonadal sex differentiation in lower vertebrates is scarce. In amphibians, GTHs do not seem to be involved in this process [10], and in mammals it is generally thought that sexualization of the CNS is a secondary event controlled by factors secreted by the already differentiated gonads [227]. In gonochoristic fish, the CNS does not seem to have a preponderant role, at least as an initial trigger, but the hypothalamo-pituitary axis is potentially active at about the time differentiation takes place and thus may be needed for the completion of the sex differentiation process. In reptiles, however, it should be noted that a hypothesis has been proposed, implicating the brain expression of aromatase enzyme in the process of gonadal sex differentiation [228]. In hermaphroditic fish species, the role of the CNS is becoming better documented. GTHs, along with upstream factors potentially regulating their synthesis and/or pituitary release, seem to play a particularly important role in triggering the sex inversion process. This could be linked with the social control of sex inversion that has been demonstrated in many species of hermaphrodites, as such a control would indeed need a brain modulation. In many lower vertebrates, steroids seem to play a crucial role in the process of gonadal sex differentiation [1, 197, 229]. More specifically, aromatase is now considered to be a key enzyme in gonadal sex differentiation, at least in reptiles [197, 229] and birds [230]. Two types of theoretical models have been proposed based either on the presence or absence of aromatase enzyme or on the androgen-to-oestrogen ratio and thus 5α-reductase-to-aromatase activity [196, 228, 229]. In fish, the importance of oestrogens in gonadal differentiation can also be considered, and the enzyme aromatase is probably one of the key enzymes needed for both ovarian differentiation in gonochoristic fish and for sex inversion in protandrous hermaphroditic fish. Despite this preponderant role of aromatase, however, results concerning 11-oxygenated androgens in fish also deserve attention, and we would like to propose a parallel hypothesis to the one suggested in reptiles [196, 228, 229]. 11-Oxygenated androgens are the active androgens in fish [231, 232]. Thus, instead of the androgen-to-oestrogen ratio adopted for reptiles, the 11-oxygenated androgen-to-oestrogen ratio in fish would direct either male (excess of 11-oxygenated androgens) or female (excess of oestrogens) differentiation. This hypothesis is in agreement with most of the information on steroids and steroid enzyme activity and/or gene expression described to date concerning fish gonadal sex differentiation.

Concerning the other factors involved in gonadal sex differentiation, a few studies have shown that the genes implicated in mammalian differentiation are also found in lower vertebrates. In that regard, Sox9 and WT1 gene products have been cloned and studied in reptiles [233] and birds [112, 234, 235], and the expression profiles of these genes during gonadal differentiation are consistent with those obtained in mammals. The anti-Müllerian hormone (AMH) complementary DNA (cDNA) has also been cloned in the chicken [236] and turtle [237], and the pattern of expression in chick gonads is strongly dimorphic during sex differentiation, as previously found in mammals [235]. Some of these genes thought to be important in the process of mammalian gonadal sex differentiation have also been found in fish, but none of them has been studied with respect to gonadal differentiation events. With the advent of molecular tools, this area will probably be investigated very soon.

Regardless of the exact nature of the social stimuli, the involvement of social factors in the initiation or modulation of sex inversion reveals the remarkable adaptive capacity of hermaphroditic species. Moreover, in at least some species such as *E. microdon* [184, 238], an individual having undergone a first sex inversion process can still present some adaptive sexual plasticity (possibility to sex reverse from male to female).

Since the first evidence of thermosensitivity in *M. menidia* [212], various studies have clearly demonstrated that environmental factors can strongly influence sex differentiation and, thus, sex ratios in gonochoristic fish. In most of the sensitive fish species examined, interactions between environmental factors and genotype have been suggested [201, 205–211, 213, 223, 224, 239]. Such environmental effects could at least partially explain some of the unexpected sex ratios recorded in fish [240]. As is seen in reptiles [197, 228], three main patterns of thermosensitivity seem to exist in sensitive fish. Contrary to reptile patterns, however, and with the exception of *O. bonariensis* [215], all-male and/or all-female populations are generally not produced at extreme temperatures; this could result from strong temperature/genotype interactions. In certain thermosensitive species, breeding pairs may thus generate highly sensitive progenies with 100% male progenies in treated groups, as has been seen for tilapia (J.-F. Baroiller and F. Clota, unpublished data). The characteristics of thermosensitivity also differ substantially from those observed in reptiles. Thus, in fish there is a large range of temperatures over which the population sex ratios fit well with a strict GSD model (this is especially true of certain thermosensitive species, such as the hirame *P. olivaceus*, which follow a male or male homogamety model [220]). Conversely, in reptiles, both sexes are only obtained within a narrow transitional range (less than 1 °C in some species) [197]. In this respect, thermosensitivity in fish resembles that in amphibians such as *Pleurodeles waltl* and *P. poireti*. In these species, genetic sex determination (male homogamety ZZ/ZW) governs sex differentiation at ambient temperatures (balanced sex ratios are observed at 16–24 °C), whereas warm and cold temperatures strongly affect gonadal sex differentiation [241]. In addition, genotype/temperature interactions are rarely reported in amphibians [241].

Several characteristics of fish sexuality are worth recalling: (i) The manipulation of the phenotypic sex differentiation in gonochoristic or the onset of sex inversion in hermaphroditic species by hormonal or environmental treatments; (ii) obtention of new viable and fertile sexual genotypes in gonochoristic species (YY, WW), and thus production of genetically all-male and all-female populations; (iii) observation of both hermaphroditic and gonochoristic species within the same family or even the same genus (for instance, *L. niloticus* is a strict gonochoristic species, whereas *L. calcarifer* is a protandrous species); and (iv) observation of both homogametic and heterogametic systems within the same genus (tilapia) or even the same species (the platyfish). All of these features make fish a tremendous model for the study of sex determination and gonadal sex differentiation in vertebrates.

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