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Gonadal differentiation and temperature effects on sex determination in the freshwater pike silverside *Chirostoma estor* Jordan 1880

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Abstract The gonadal description of the freshwater atherinopsid pike silverside *Chirostoma estor* suggests that the gonads differentiate as ovaries or testes by 8 weeks after hatching when raised at 21 °C. Thermal treatments at 14 °C, 21 °C and 29 °C applied from fertilisation, clearly affected phenotypic sex ratios, suggesting that the thermolabile window of sex determination occurred early in development. In this study, exposure to the highest temperature led to male-biased sex ratios in this species. However, the effects of the lower and medium temperatures on the sex ratios were less clear, suggesting the presence of a mixture of genotypic and temperature-dependent sex determination (TSD) mechanisms in *C. estor*, similar to other atherinopsids. This work further enhances our knowledge regarding the diversity and plasticity of TSD mechanisms in atherinopsid and teleost fish.

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Introduction

The sex determination process in vertebrates, including fish, is driven by genotypic and environmental factors during the early stages of development, and the degree to which these factors influence each species may vary even in closely related species (Strüssmann and Patiño 1999; Bull 2008). Thus, sex-determining mechanisms can be broadly classified as genotypic (GSD) or environmental (ESD) as the extremes of a continuum (Strüssmann and Patiño 1999). Among the ESD forms, temperature-dependent sex determination (TSD) is the most commonly investigated process (Valenzuela et al. 2003), and it has been reported in over 13 families of fish (Ospina-Álvarez and Piferrer 2008). The coexistence of both GSD and TSD have been recently characterised in the pejerrey *Odontesthes bonariensis* (Yamamoto et al. 2014).

Sex determination is controlled by the activities of numerous molecular and biochemical pathways (e.g., transcription factors, steroidogenic enzymes, receptors, second messenger systems), leading to the syntheses of specific proteins that are responsible for the differentiation of either the ovary or the testis (Devlin and Nagahama 2002).

At the molecular level, several transcription factors have been recognised as key sex-determining genes,

such as *SRY* in eutherian and metatherian mammals (Sinclair et al. 1990; Foster et al. 1992), *DMY/dmrt1bY* in the teleost *Oryzias latipes* (Temminck and Schlegel 1846) (Matsuda et al. 2002; Nanda et al. 2002); *DM-W* in the amphibian *Xenopus laevis* (Daudin 1802) (Yoshimoto et al. 2008) and *DMRT1* in the domestic chicken *Gallus gallus* (L., 1758) (Smith et al. 2009). Additionally, four genes that are not transcription factors have been described to play key roles in fish sex determination; A) the Y-linked anti-Müllerian hormone (*amhy*) in the Patagonian pejerrey *Odontesthes hatcheri* (Eigenmann 1909) (Hattori et al. 2012); B) the anti-Müllerian hormone receptor type II (*Amhr2*) in the tiger pufferfish *Takifugu rubripes* (Temminck and Schlegel 1850) (Kamiya et al. 2012); C) sexually dimorphic on the Y chromosome (*sdY*), which is an immune-related gene, in the rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) (Yano et al. 2012); and D) the gonadal soma-derived growth factor on the Y chromosome (*Gsdf^Y*) in the medaka *Oryzias luzonensis* (Herre and Ablan 1934) (Myosho et al. 2012).

More recently, epigenetic mechanisms on sex determination have been demonstrated in fish (Navarro-Martín et al. 2011) and in turtles (Matsumoto et al. 2013), in which high degrees of methylation have been observed in the promoter of the *cyp19a* gene following exposure to high temperatures, leading to male-biased sex ratios in the offspring. These cases illustrate the diversities of the sex determination mechanisms that are present in teleost fish. However, the precise molecular interplay and metabolic pathways underlying the genotypic and environmental factors affecting the sex differentiation processes in fish and in other non-mammalian, poikilothermic species remain unclear (Ospina-Álvarez and Piferrer 2008; Guerrero-Estevez and Moreno-Mendoza 2010; Hattori et al. 2012).

Species with TSD have been proposed to be reliable indicators of the biological effects of global warming because temperature-induced sex ratio shifts constitute direct fitness responses to thermal fluctuations, and subsequently, to population dynamics (Janzen 1994). Thus, atherinopsid fish have recently become interesting model organisms because they present several patterns of TSD mechanisms and marked reproductive responses to temperature shifts (Strüssmann et al. 2010). For example, masculinisation is observed at high temperatures in the following atherinopsids: *Menidia menidia* L. 1766 (94 % males at 28 °C), *M. peninsulae* Goode and Bean 1879 (74 % at 32 °C) (Conover and Kynard 1981; Conover and Heins 1987; Yamahira and Conover 2003) and *Odontesthes bonariensis*, in which the most pronounced effects have been observed (up to 100 % males at 29 °C) (Strüssmann et al. 1996a, 1997).

The endemic Mexican freshwater pike silverside *Chirostoma estor* is an endangered atherinopsid species with critically reduced wild populations primarily due to pollution, overfishing and a lack of conservation programs (Martinez-Palacios et al. 2006). Since 1999, studies have been conducted to optimise aquaculture protocols to ensure the survival of this species, which has been shown to have high docosahexaenoic acid content (Fonseca-Madrigal et al. 2012; Fonseca-Madrigal et al. 2014). This work aimed to describe early gonadal development and differentiation and the effects of water temperature on these processes.

Methods

Rearing conditions

All experiments were performed at the aquaculture laboratory facilities at the Instituto de Investigaciones Agropecuarias y Forestales of Universidad Michoacana de San Nicolás de Hidalgo in Morelia, Mexico. The experimental system consisted of nine 60-l aquarium tanks (three per replicate) attached to mechanical, biological and UV filtration with water flow rate of 0.048 l s^{-1}. Automatic temperature control was provided by an aquarium heater and chiller (LN-5800, BOYU, Raoping Guangdong, China) used throughout the system, which maintained temperatures at ±0.5 °C at all times.

A completely closed water recirculation system was used, which included 5 g l^{-1} of salt (Martinez-Palacios et al. 2004) and 12 h light/12 h dark photoperiods provided by fluorescent commercial bulbs (750 lm, 6500 K). Oxygen was maintained at approximately 6 g l^{-1}, pH 7–8, and NH$_3$, NH$_4$, NO$_2$ were maintained at safe levels.

Water quality was monitored in the system throughout all the experiments as follows: salinity was assessed with a refractometer (S/Mill-E 0–100 g l^{-1}, ATAGO, Bellevue, WA 98005 USA); temperature was monitored with a mercury thermometer (−20 °C to 110 °C); pH, NH$_3$, NH$_4$ and NO$_2$ levels were evaluated with a color-imetric test kit (FF-3, HACH, Loveland, CO 80539 USA); and dissolved oxygen levels were monitored

with an oxymeter (51B, YSI, Yellow Springs, OH 45387 USA).

Experiment 1. Early gonadal differentiation

To describe and to define the characteristics of early gonadal differentiation, approximately 1000 eggs were obtained from three different pairs and manually fertilised. Then, pooled eggs were divided in replicates ($n = 3$), incubated and reared at 21 °C until 12 weeks after hatching (wah). The fish density was adjusted to 4 larvae per litre. Larvae were fed live *Brachionus plicatilis* Müller 1786 and *Artemia spp.* (Leach 1819) nauplii ad libitum throughout the experiment according to Martinez-Palacios et al. (2006). In total, 20 larvae from each replicate was sampled at one, four, eight and 12 wah, sedated with ice water and culled as previously described for other atherinopsid species (Hattori et al. 2009). The total length and mass of each individual larva were measured, and the trunk was dissected and fixed for further histological analysis.

Experiment 2. Effects of temperature treatment applied from fertilisation to four wah on sex ratios

In this experiment, 2500 eggs from three different broodstock pairs were manually stripped, fertilised at 23 °C and kept separate to assess the parental effects on the offspring sex ratios. The fertilised eggs from each pair were divided into three thermic treatment groups; 14 °C, 21 °C and 29 °C ($n = 3$). After four wah of thermal exposure, the fish from the 14 °C and 29 °C treatments were gradually acclimated (1 °C h^{-1}) to the control temperature (21 °C) and maintained for further growth to 12 wah. The fish density at the start of the trial was 3 larvae per litre, and the feeding was performed as described in Exp. 1. At the end of experiment the mean final mass was recorded from 40 larvae in the 14 °C treatment, 60 larvae from the 21 °C and 60 larvae of 29 °C treatments, these samples were randomly obtained following the procedure described in Exp. 1. The hatching and survival rate were calculated with the entire population at the beginning and the end of the trial.

In this experiment, the trunk was also dissected individually for a histological assessment to determine the phenotypic sex ratio and thermal sex ratio differences between broods.

Experiment 3. Effects of temperature treatment applied from hatching to four wah on sex ratios

For this experiment, approximately 5000 eggs pooled from ten different broodstock pairs were manually stripped, fertilised and incubated at 23 °C. Once hatched, the fish were divided into three thermal treatments, including 19 °C, 23 °C and 27 °C, with their respective replicates ($n = 3$). The thermal treatments ended at four wah, and then, all the treatments were acclimated to 23 °C (control temperature). This temperature was maintained until 12 wah for continued growth. The fish densities and acclimatisation processes were performed as described in Exp. 2. The fish were fed *B. plicatilis* and *Artemia sp.* nauplii ad libitum during the first 4 weeks and were fed a commercial microdiet (Otohime S-1, Nihombashi Muromachi, Chuo-Ku, Tokyo, 103–0022 Japan) at a daily rate of 3 % of biomass for the last 8 weeks. The fish were sampled at the end of experiment as previously described in Exp. 2.

Histological analysis of gonad differentiation

All the samples were fixed in Bouin's solution (HT10132, SIGMA, www.sigmaaldrich.com) for 24 h and stored in 70 % ethanol. Then, the samples were dehydrated in an ascending ethanol series and embedded in paraffin enriched with highly pure polymers (K93091409-Histosec, MERCK KGaA, Darmstadt, Germany). Six-micron cross sections were prepared using a microtome (Jung-Histocut 820, Leica, Leider Lane, Buffalo Grove, IL 60089 USA), which were mounted onto glass slides for haematoxylin-eosin staining (Hattori et al. 2009). The preparations were observed under a light microscope, and phenotypic sex was determined according to the criteria used for other atherinopsids as described by Strüssmann et al. (1996b, 1997).

Statistical analyses

All the data were analysed using the Sigma Plot v.11.00 software (Systat Software, Inc., 1735 Technology Drive, Suite 430 San Jose, CA 95110 USA). The statistical significances of the hatching rates, survival rates, and phenotypic sex ratios between the temperatures and between the broods were determined by the X^2 (99 % C.I.) test. The mean final mass data sets were tested for normality (the Kolmogorov-Smirnov test) and equal

variance among groups (Bartlett's test). Then, the normally distributed data sets were analysed using one-way ANOVA (95 % C.I.) with post hoc Tukey's test for multiple comparisons. Non-parametric data sets were analysed using the Kruskal-Wallis test, followed by post hoc Dunn's test to establish significance between the treatment groups. The significance level was set to $p \leq 0.05$ for all analyses, except for the X^2 analysis, for which $p \leq 0.01$.

Results

Gonadal description

In Exp. 1, all the one-wah larvae presented early, undifferentiated, rounded gonads containing somatic and primordial germ cells (PGCs) (Fig. 1(a)). At four wah, two clearly identifiable morphologies of different sizes were found; small undifferentiated gonads containing somatic cells and PGCs similar to those found at one wah (Fig. 2(a)) and larger gonads with developing ovaries, characterised by observable active PGC proliferation, meiotic cells and somatic cell cluster outgrowths in the distal region, which appear very early in the ovary (Fig. 1(b)) forming the ovarian cavity in latter stages (Fig. 1(c)).

Thereafter, the gonads possessing either testicular or ovarian structures could be clearly identified at eight wah. The developing testes contained small blood capillaries close to the proximal-lateral regions, few spermatocysts, somatic cells, and apparent primary sperm ducts (Fig. 2(b)), whereas the developing ovaries presented a clear ovarian cavity, oogonia, large blood capillaries, and smaller capillaries in the proximal-lateral regions (Fig. 1(c)).

At 12 wah, the developing testes contained primary spermatocytes, several spermatocysts and testis lobules, and the blood capillaries were located on the opposite sides of the spermatocysts, close to the primary sperm ducts and to the external testicular wall (Fig. 2(c)). In contrast, the developing ovaries included diplotene and perinucleolar primary oocytes, and the blood capillaries appeared close to the ovarian outer wall in the proximal-lateral regions (Fig. 1(d)).

In Exps. 2 and 3, each female displayed either phase I ovaries, which are characterised by oogonia and pachytene and diplotene oocytes (Fig. 3(b)), or phase II ovaries, which are characterised by oogonia and pachytene, diplotene and perinucleolar oocytes (Fig. 3(a)), the temperature determined the ovaries size among treatments, finding the smallest ovaries at 14 °C, and largest ovaries at 21 and 29 °C. The 100 % of ovaries of fish raised at 21 and 29 °C were found at phase II (Fig. 3(a)), however at 14 °C only the 84 % of ovaries presented phase II and the 16 % of ovaries were found at phase I (Fig. 3(b)). In Exp. 2, males raised at 21 °C and 29 °C had well developed testes with spermatogonia, spermatocysts, primary spermatocytes, spermatic ducts and somatic cells (Fig. 3(c)); however, the gonads of male fish raised at 14 °C in the same experiment had less developed testicular lobules (Fig. 3(d)). Histological similarities were observed in the male fish that were raised at 19 °C, 23 °C and 27 °C in Exp. 3.

Hatching rates, survival rates and mean final masses

In Exp. 2, the hatching and survival rates at 14 °C, 21 °C and 29 °C significantly differed among all treatments within each brood. For the three broods the lowest and highest hatching and survival rates were found at the lowest temperature (14 °C) and at 21 °C, respectively,
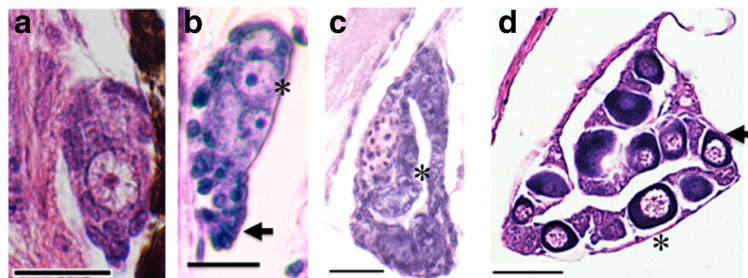

Fig. 1 Early ovary development of *C. estor* larvae at 21 °C (experiment 1). **a** Undifferentiated gonad at one wah; **b** Ovarian differentiation at four wah, indicated by cluster of somatic cells (*arrow*) and meiotic prophase cells (*asterisk*); **c** Ovarian differentiation at eight wah, presence of ovarian cavity formed (*asterisk*); **d** Ovary at 12 wah, presence of perinucleolar oocytes (*arrow*), oogonia (*asterisk*); *bars* represent 10 (**a**, **b**), 20 (**c**) and 50 (**d**) microns

Fig. 2 Early testis development of *C. estor* larvae at 21 °C (experiment 1). **a** Undifferentiated gonad at four wah; **b** Testicular differentiation at 8 wah, presence of main sperm duct (*asterisk*) and spermatocyst (*arrow*); **c** Testis at 12 wah, presence of testis lobule (*dashed arrow*); *bars* represent 10 (**a**) and 20 (**b**, **c**) microns
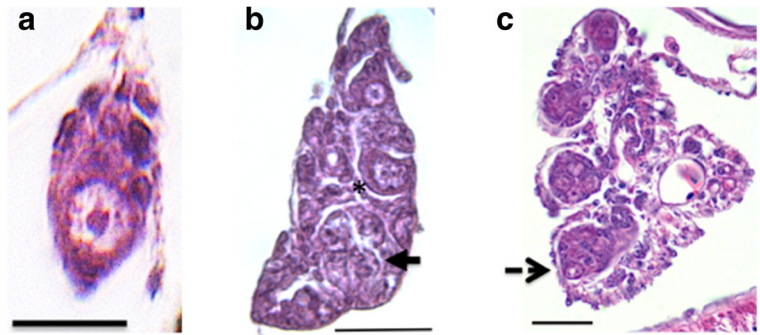
except for the first brood, which showed the better hatching rate was at 29 °C. Regarding the final mass a clear pattern appears, in which the weight gain increased proportionally to temperature (Table 1).

In Exp. 3, the survival rates and mean final mass did not differ significantly between the 27 °C and 23 °C treatments. However, significant differences in the survival rates and mean final mass were found between these treatments and that of 19 °C (Table 1).

Effects of temperatures on sex ratios

In Exp. 2, the sex ratios (male proportions) of the brood of each pair were analysed. The first brood displayed significant differences only at 29 °C compared to 14 °C and 21 °C (Table 1). In the second brood, no significant differences were observed between any of the treatments. Finally, significant differences were found among all treatments for the third brood. At 14 °C the percentage of males was 48 %, at 21 °C was 75 % and for 29 °C was 92 %, showing a clear masculinization response as temperature increases (Table 1). The mean proportions of males analysed combining the results of the three parental lines were 57.33 % (14 °C), 65 % (21 °C) and 82 % (29 °C), and significant differences were observed among treatments. In Exp. 3, the proportions of male fish did not significantly differ among any of the treatments.

Fig. 3 Histological appearance of phase II (**a**) and phase I (**b**) ovaries and testes (**c**, **d**) at 12 wah day old juveniles reared at 29 °C (experiment 2; **a**, **c**) and 14 °C (experiment 2; **b**, **d**), *bars* represent 50 (**a**) and 20 (**b**, **c**, **d**) microns
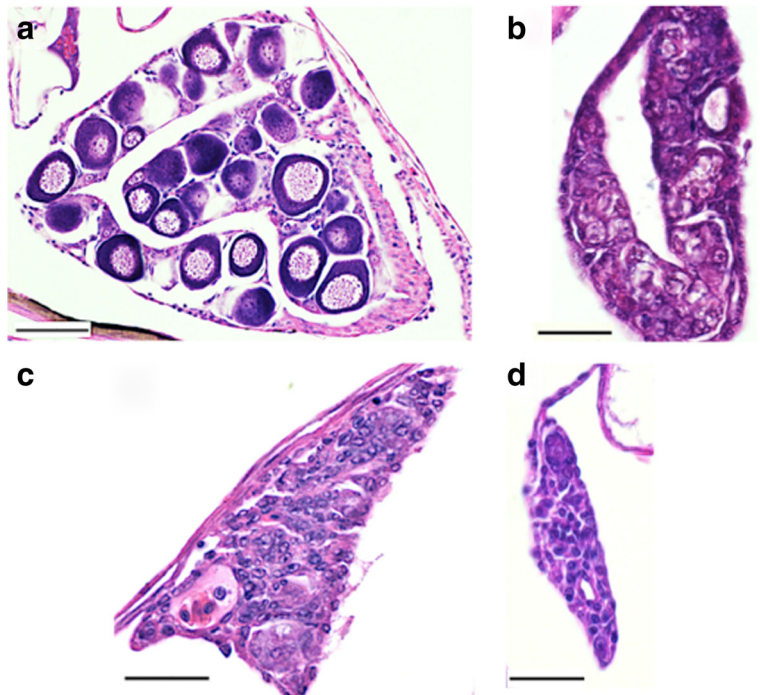
Table 1 Hatching and survival, rates, mean final mass, and sex ratios of *C. estor* incubated and reared at different temperatures before and after hatching (experiment 2) or after (experiment 3) hatching

n number of fish by temperature in each brood, °C Degree centigrades, *mg* milligrams; (a, b, c) different letters indicate significance differences between treatments for each brood in the same measurement column. Sex ratio is expressed as % of male proportions

Experiment	Brood	Temperature (°C)	Hatching rate (%)	Survival rate (%)	Mean final mass (mg)	Sex ratio (%)	(n)
2	1	14	30.4[a]	7.8[a]	216.67 ± 57.7[a]	67[a]	12
	1	21	52.4[b]	91.6[b]	555.0 ± 82.5[b]	65[a]	20
	1	29	60.8[c]	37.1[c]	680.0 ± 226.1[c]	90[b]	20
2	2	14	16.0[a]	14.5[a]	257.14 ± 78.6[a]	57[a]	7
	2	21	40.0[b]	83.3[b]	640.0 ± 131.3[b]	55[a]	20
	2	29	13.0[a]	38.4[c]	750.0 ± 191.1[c]	64[a]	14
2	3	14	42.0[a]	25.0[a]	252.38 ± 51.1[a]	48[a]	21
	3	21	69.0[b]	81.1[b]	545.0 ± 139.4[b]	75[b]	20
	3	29	41.0[a]	46.3[c]	723.0 ± 133.5[c]	92[c]	26
3	4	19		29.3[a]	218.43 ± 10.3[a]	40[a]	40
	4	23	78.5	47.2[b]	266.02 ± 12.2[b]	52[a]	60
	4	27		48.1[b]	275.96 ± 13.1[b]	50[a]	60

Discussion

Atherinopsid fish have become interesting models for studying the mechanisms underlying temperature-dependent sex determination in vertebrates due to the high thermal sensitivity of gonadal development observed in these organisms at both high and low temperatures. The identification of the Y-linked *amhy* gene in *O. hatcheri* and more recently the confirmation of the coexistence of genotypic and environmental mechanisms of sex determination in *O. bonariensis* has further increased the interest of this group of fish for investigating TSD in a GSD context (Hattori et al. 2012; Yamamoto et al. 2014). In this study, we first described the histological differentiation of the gonads in *C. estor* raised at 21 °C for 12 wah. Then, we determined the effects of low, intermediate and high temperatures applied before and after hatching on phenotypic sex ratios.

Histological analyses during gonad differentiation revealed a clear gonochoristic differentiation pattern in *C. estor*, in which ovarian differentiation occurred between one and four wah, whereas testicular differentiation occurred later, between four and eight wah. These findings are similar with other atherinopsid (*O. hatchery*) in which the first features of ovarian differentiation appear at three to four wah, and testicular differentiation begins at five to six wah (Hattori et al. 2012). In fact, in most gonochoristic teleosts, ovarian differentiation occurs first (Strüssmann and Nakamura 2002). Developing ovaries presented a central ovarian cavity, with oogonias and primary oocytes between the cavity and the ovarian wall or visceral peritoneum. This pattern differs from that described previously for other atherinopsids, such as *O. bonariensis*, *O hatcheri* and *O. argentinensis* (Strüssmann et al. 1996a, b, c, 1997), in which the ovarian cavity is located along the ventral region of the gonad, with follicle of germ cells distributed on the opposite site, i.e., along dorsal region. Regarding the testes of *C. estor*, it showed external digitiform projections or microlobules along each pair of lobules and blood vessels on the opposite side of mesorchia. This feature has not been observed in other atherinopsids, in which the blood vessels are close to the mesorchia and the lobules are smooth (Strüssmann et al. 1996a, b, c, 1997).

In thermal treatment experiments, we observed balanced phenotypic sex ratios for temperature exposure after hatching. However, when fish were exposed to similar thermal treatments shortly after fertilisation until four wah, sex ratio shifts toward males were observed at high temperature, which was suggestive of TSD-related effects. In this study, the proportions of phenotypic males obtained from broods 1 and 3 (90 % and 92 %, respectively) at 29 °C were significantly higher compared to those at the intermediate temperatures (65 % and 75 %, respectively) (Table 1). Similar findings have also been observed in other atherinopsid species (Conover and Heins 1987; Strüssmann et al. 1996a, 1997; Yamahira and Conover 2003). On the other hand, low temperature (14 °C; Exp. 2) did not have feminising effects, as opposed to the patterns of *O. bonariensis*, *O. hatcheri* and *M. peninsulae*, for which feminisation rates of 100 %, 89 % and 90 %, respectively, have been

reported (Strüssmann et al. 1996a, 1997; Yamahira and Conover 2003). Feminization at low temperatures seems not to be a general response among teleosts, likewise masculinization at high thermal regimes (Hattori et al. 2007; Ospina-Álvarez and Piferrer 2008). Nevertheless, we have to consider the possibility of feminization at even lower temperatures than that used in this study. Another possibility is that another unknown stressor (s) able to increase cortisol levels (e.g., salinity, background color, light intensity) (Hattori et al. 2009) could be acting on sex determination resulting in balanced sex ratios instead of female-biased ones at low temperature.

Although the analyses of body weight as well as the rates of hatching and survival indicate that the lowest and the highest temperatures are close to the extreme thermal range tolerated by C. estor and that intermediate ones are within the optimal range for this species, according to the reported previously for this species (Martinez-Palacios et al. 2002), temperatures even lower or higher could be explored for a shorter period in future studies in order to achieve higher sex reversal rates. Shortening this period are important in breeding programs once long-term treatments at high temperatures may have chronic effects on juvenile-adult performance impairing gametogenesis and causing sterility, as reported in O. bonariensis (Ito et al. 2008).

As regards to the timing of sex determination, we propose that the thermolabile window of sex determination commences somewhere after fertilisation, i.e., during embryogenesis. This pattern resembles that reported in O. hatcheri (Strüssmann et al. 1996a), in Oryzias latipes (Hattori et al. 2007) and in Oreochromis niloticus (Rougeot et al. 2008) and differs from that reported in O. bonariensis, in which the thermolabile window has been estimated to occur after hatching. This assumption, coupled to the fact that high temperature in C. estor did not induce 100 % masculinisation or that low temperatures were ineffective may suggest that this species presents strong GSD as in O. hatcheri (Strüssmann et al. 1997; Hattori et al. 2012), controlled by a genotypic sex determinant. The identification of such determinant as well as the transcription profiling of sex-related genes could clarify the sex determining mechanisms and the gonad differentiation process in this species, which in turn, could be used as important genetic tools in reproductive studies of this species.

Our results on C. estor sex determination using different broods support the idea that the degree or strength of GSD and TSD are highly dependent on species or even on broods (Ospina-Álvarez and Piferrer 2008). In this study, such variations were evident when larvae from different pairs were analysed. These sex ratio differences may be due to differences in thermal sensitivity between each pair and thus between each brood, affecting the responses of phenotypic sex ratios to the same temperature.

In conclusion, this study indicates that high temperature during early development can affect sex ratios in C. estor, reinforcing the idea of TSD as a widespread sex-determining mechanism among atherinopsid fish. The presence of TSD in C. estor suggest that sex ratios of wild populations as well as its dynamics, may also be affected in a global warming or climate change scenario as previously suggested for silversides by Strüssmann et al. (2010), mainly because C. estor is known to spawn in shallow areas that may be more prone to high temperatures. Additionally, TSD could be further explored as a biotechnological tool for sex control technology in aquaculture programs or restocking management of this landlocked species.

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