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Post-larval exposure to warm temperature enhances female ratio, while starvation and photoperiod do not: The case of European seabass, Dicentrarchus labrax

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Abstract :

One of the primary challenges in European seabass farming is optimizing the number of females, which grow faster and mature later than males. However, typical rearing practices often result in populations with a high proportion of males. To test whether photoperiod, temperature and fasting impact the sex ratio, two distinct experiments were conducted. In the first one, we tested the effects of two photoperiods (12 L:12D vs 10 L:14D), applied until 90 dph at 16 °C. Then, on the same fish, photoperiod was set to 12D:12 L and four different temperature treatments (19 °C, 21 °C, 23 °C, and 25 °C) were applied from 90 to 160–192 dph. In a second experiment, we tested the impact of fasting at four different times during the sex determination window: from 60 to 65 dph; 81 to 87 dph; 102 to 110 dph and 123 to 133 dph, at a temperature of 21 °C. Neither photoperiod (in the first experiment) nor starvation during larval development (in the second experiment) altered sex ratio. Conversely, we revealed (in the first experiment) that the proportion of females increased with post-larval rearing temperature: 30.4% at 19 °C, 36.3% at 21 °C, 44.1% at 23 °C, and 48.7% at 25 °C. Thus, late warm thermal treatments, following an initial cold treatment at 16 °C until 90 dph, can be useful to compensate the deficit of females in farmed sea bass production.

Highlights

► Late high temperature can yield females in seabass. ► This can be applied to improve seabass production ► We found no effect of photoperiod or starvation on sex ratio.

Keywords : Sex determination, Aquaculture, TSD, ESD

Introduction

The European seabass (*Dicentrarchus labrax*) is an important species for European mariculture (Vandeputte et al. 2019). Over the last decade, seabass aquaculture has increased to reach 98% of the total production, with only 2% remaining from capture fisheries (FAO Fish Stat 2020). Despite the large increase in production, several aspects of European seabass farming require further improvement, such as the control of sex ratio. Sexual dimorphism is frequent in farmed fish, with zootechnical characteristics (growth, appearance, processing yields, caviar production …) more favorable in one sex than in the other. In the European seabass, there is sexual dimorphism for growth, with females growing faster and maturing later than males, making them more profitable for farming. Still, sex ratio is not yet properly controlled under farming conditions, where the proportion of males can reach 70 or even 100% (Vandeputte and Piferrer, 2019). Controlling sex ratio requires the knowledge and understanding of the mechanisms of sex determination and sex differentiation. Many studies investigated such issues, showing that the European seabass possesses a polygenic sex determination system with an environmental influence (Piferrer et al., 2005; Vandeputte et al., 2007; Geffroy et al. 2021a). Still, methods to produce more females in a repeatable way are not completely satisfactory. As in many fish species, temperature plays a key role in determining the sexual fate of European seabass (Piferrer et al., 2005) in which the thermosensitive period is generally thought to occur a few days to weeks after hatching (Baroiller et al. 1999). In most fish species with a temperature dependent sex determination (TSD), early exposure to high temperature promotes masculinization (Ospina-Alvarez and Piferrer, 2008; Geffroy and Wedekind, 2020). In European seabass, it has been shown that high temperature experienced by the fish during their early life indeed masculinizes populations by decreasing *cyp19a* mRNA expression levels in the female gonads through the methylation of its promoter (Navarro-Martin et al. 2011). Conversely, an exposure to cold temperature (<17 °C) until metamorphosis (55-66 days post fertilization) promotes the development of the female phenotype (Pavlidis et al., 2000; Navarro-Martin et al. 2009; Navarro-Martin et al. 2011; Sfakianakis et al., 2013; Vandeputte and Piferrer 2019). However, a recent study demonstrated that long-term (up to 244 days post hatching, dph) exposure to a low temperature (16 °C) biases European seabass sex ratio towards males (Vandeputte et al. 2020). We may then hypothesize that there may be two distinct thermosensitive periods in the European seabass, as a cold temperature treatment applied before metamorphosis has a feminizing effect, while it has a masculinizing effect at later stages. Consequently, we may wonder whether warm temperature applied after metamorphosis may feminize sea bass populations.

Warm temperature is also known to increase metabolism and therefore affect the energy balance in ectotherms. It is thus possible that the energy available at a specific time in larval development could be the real factor influencing sex determination, as shown in some species of nematodes, insects, amphibians, reptiles and fish (Geffroy 2022). For example, early starvation of medaka (*Oryzias latipes*)

induced sex-reversal of genetic females into phenotypic males (Sakae et al. 2020). In the European seabass, females tend to have a higher energy content than males very early in development (Geffroy et al. 2021a) and the proportion of females is higher in sea bass lines selected for growth (Geffroy et al. 2021b). Hence, whether energy intake can affect the sex of individuals is also a question worth investigating.

Finally, among the environmental factors involved in sex determination, photoperiod is known to have an effect on some invertebrates like insects and crustaceans (Korpelainen 1990). In certain fish species, such as the California grunion *Leuresthes tenuis*, a combined effect of photoperiod and temperature on sex determination has also been observed (Brown et al. 2014). Continuous light exposure also triggered masculinization in the pike silverside *Chirostoma estor*, likely through the activation of the stress axis (Corona-Herrera et al. 2018). In the only published attempt to test the effect of photoperiod on the sex ratio of the European seabass, no significant effects were detected (Blasquez et al 1998), however this experiment was conducted at a masculinizing temperature (20-24 °C), thus potentially hiding an effect of photoperiod.

In order to refine our knowledge on the effects of the environment on sex determination in the European seabass, we tested if three specific treatments could affect the sex ratio of European seabass by exposing fish to: 1) two distinct photoperiod treatments (12L:12D and 10L:14D) applied during the early larval phase (from 9 to 90 dph), at a common temperature of 16°C, which is necessary to enable female sex determination in the species (Navarro-Martin et al., 2009); 2) four temperatures (19; 21; 23 and 25 °C, in the range of natural summer temperature in sea bass native range) applied on the same fish from 90 dph until 160-192 dph, at a common 12L:12D photoperiod and 3) in a second experiment, four distinct fasting periods applied at different times (between 60 and 133 dph).

Material and methods

Ethical statement

All experiments were conducted in the Experimental Marine Aquaculture platform of Ifremer at Palavas-les-Flots, France, accredited to use and breed laboratory animals (n°C341926). Experiment 1 and Experiment 2 were evaluated by the Ethical Committee n° 036 and authorized by the French Ministry of Higher Education, Research and Innovation (Experiment 1, authorization number APAFIS#24426-2020022118172785; Experiment 2, authorization number APAFIS#19676-2019021915002143). All experimental procedures were conducted following the guidelines for animal experimentation established by Directive 2010-63-EU of the European Union and the corresponding French legislation.

Experiment 1: photoperiod and temperature

Production of fish

The fish population used came from a complete factorial mating with 10 males and 8 females from a wild western Mediterranean strain of the European sea bass *Dicentrarchus labrax,* performed by artificial fertilization as described in Grima et al. (2010). Fertilized eggs were incubated at 14 °C until 72 hours post fertilization (hpf) and then evenly dispatched to six tanks of 500 L each, in which temperature was progressively increased to 16 °C until hatching. Fish were fed on *Artemia* nauplii for the first 40 days and then weaned on a commercial European seabass feed (Marin Start, Le Gouessant, Lamballe, France). Salinity was 25 psu from hatching to 48 dph, and then natural salinity (37-41 psu) was applied for the whole experiment. Dissolved oxygen was maintained at 100% saturation throughout the experiment.

Photoperiodic treatments

From 9 to 90 dph, three tanks were submitted to a photoperiod of 10L:14D, which is representative of January-like conditions in the western Mediterranean, and three other tanks were submitted to a 12L:12D regime, which is representative of March-like conditions in the same area.

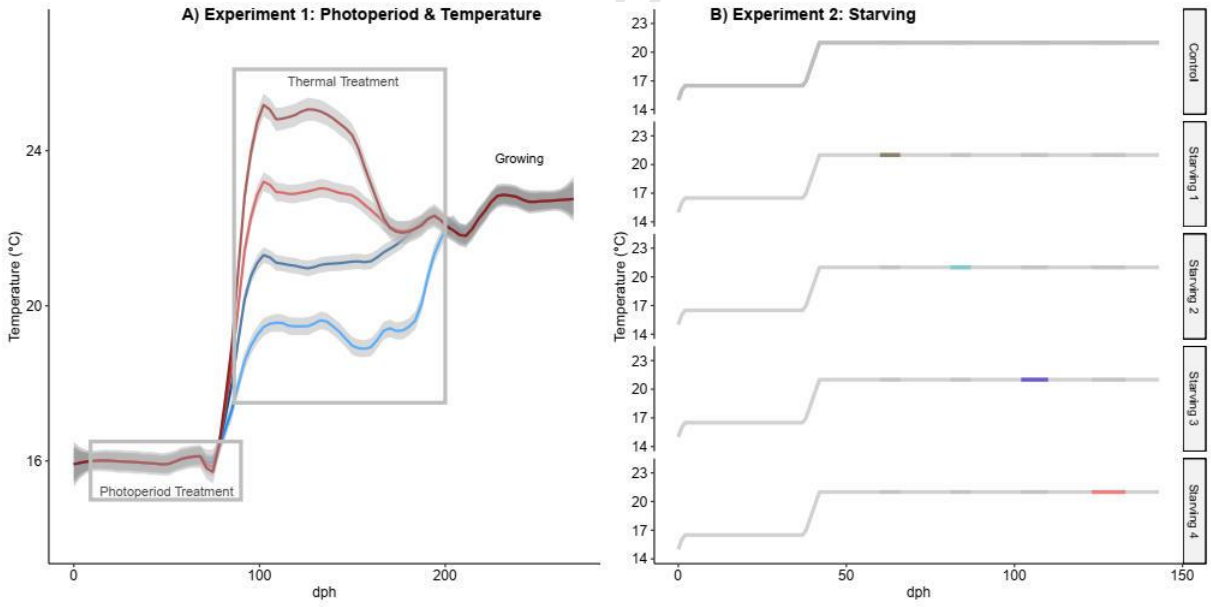

Figure 1 Schedule (dph) and temperatures (°C) of experiment 1 (A) and experiment 2 (B).In experiment 1, half of the fish were subjected to a 12D:12L photoperiod treatment and half of the fish to a 10L:14D photoperiod treatment from 9 to 90 dph. Colour bars in panel B highlight the timing of starvation treatments.

Thermal treatments

Temperature was set to 16 °C until 85 dph (Figure 1A). From that point, temperature was gradually increased, reaching 19 °C at 87 dph; 21 °C at 88 dph; 23 °C at 89 dph and 25 °C at 90 dph (Figure 1A). At 87 dph, fish transfer for the first thermal treatment was performed. Fish (n=80) from each of the three tanks previously submitted to a same photoperiod were transferred to two duplicate tanks of 110

L each in a recirculating system where temperature was set to 19 °C. This system consisted of four tanks: two containing 240 fish each initially treated with a short (10L:14D) photoperiod, and two containing 240 fish each initially treated with a normal (12L:12D) photoperiod. The same procedure was applied during the following three transfers at 88, 89, and 90 dph to the other three recirculated systems in which temperature was set to 21, 23, and 25 °C, respectively.

In the four temperature treatments, a photoperiod of 12L:12D was applied. Thermal treatments were applied until the fish reached 8 cm fork length, at the thermal age of 1500 DD_{10} (day-degrees with at 10 °C reference), a stage at which sex is definitively fixed (Saillant et al., 2003; Piferrer et al., 2005). This happened at 160 dph for the 25 °C treatment, and at 161, 174 and 192 dph for the the 23, 21, and 19 °C treatment groups, respectively. At the end of each thermal treatment, all fish were individually identified with RFID glass tags (Biolog-ID), then weighed and measured before being transferred and pooled in a bigger tank of 1500 L in a recirculating system where temperature was set to 22-23 °C, until they were sexed at 409 dph. .

Sexing

At 409 dph, all fish (n=1926) were euthanized by an overdose of anaesthetic (Benzocain, 150 mg/l). Each fish was sexed by *in situ* macroscopic examination of the gonads, which is quite easy at this age, or, in rare cases of hesitation, by microscopic observation of gonadal squashes (Menu et al, 2005).

Experiment 2: fasting

Production of fish

The fish population used originated from a complete factorial mating using artificial fertilization between 11 males and 10 females from the third generation of a growth selected line based on the western Mediterranean sea population of European seabass, following Grima et al. (2010). Fertilized eggs were incubated at 14 °C until 72 hpf and then evenly dispatched in two tanks of 500 L each, in which temperature was progressively increased to 16.5 °C until hatching (hatching rate was 89%). Fish were maintained at 16.5 °C until 36 dph, at which point temperature was increased by one degree per day until reaching 21 °C, and then maintained throughout the experiment. At 54 dph, 4500 larvae from the two tanks were equally distributed into 15 110L-tanks (300 larvae per tank). Fish were fed *Artemia* nauplii from 10 to 50 days after hatching, then weaned onto a commercial European seabass diet (Marin Start, Le Gouessant, Lamballe, France).

Starving period

Larvae were exposed to different treatment conditions in triplicates (3 tanks per condition): Starving 1: fasting for 5 days (from 60 to 65 dph); Starving 2: fasting for 6 days (from 81 to 87 dph); Starving 3: fasting for 8 days (from 102 to 110 dph); and Starving 4: fasting for 10 days (from 123 to 133 dph)

(Figure 1B). Three tanks remained as control tanks (i.e., normal feeding regime). The duration of fasting periods for each group were calculated based on the dynamic energy budget (DEB) of European sea bass (Stavrakidis-Zachou et al. 2019) to ensure fish will start autophagy, without triggering mortality. On the day following the end of each fasting period, the length and weight of 10 fish from each control tank (n = 30) and 10 fish from the corresponding treatment tank (n = 30) were recorded to track real weight loss. Following the last sampling at 134 dph, all remaining juvenile fish were transferred to 1500 L-tanks, and maintained at 21 °C until sexing at 393 dph via *in situ* morphological analysis of the gonads.

Data analysis

Differences in sex ratio among treatments were assessed with a generalized linear model with mixed effects, with a logit link function using the lme4 package in R (Bates et al, 2015). For Experiment 1, photoperiod and thermal treatment were considered fixed factors, while tank within treatment was treated as a random factor. Post-hoc differences among treatments were tested by multiple comparisons of means with Tukey adjustment. A similar approach was performed for assessing the effect of starvation on sex ratio in the Experiment 2. We also tested if the final weight and length of fish experiencing early starvation differed according to starvation treatment using a linear mixed model in R with package lme4, with treatment as a fixed effect and tank as a random effect. Results are given as mean ± standard error (se). All plots were designed using the ggplot2 package.

Results

In Experiment 1, there was no effect of the photoperiod treatment on sex ratio ($P>0.5$). Conversely, we detected a highly significant effect of the thermal treatment (F-value = 11.5; $P<0.001$), with significantly more females produced at 25 °C (48.7±3.6%) than at 21 (36.3±0.7%) and 19 °C (30.4± 2.0%) (Figure 2A). Significantly more females were also produced at 23 °C (44.1±3.0%) than at 19 °C (Figure 2A). The difference in sex ratio between 23 and 21 °C almost reached significance ($P=0.053$).

Thanks to individual tagging between 160 and 190 dph (depending on the treatment), individual growth was monitored until sexing at 409 dph. Fish treated at 23-25 °C were larger than those treated at 19-21 °C, regardless of their sex (Table S1). However due to the fact that we pooled replicates after tagging, the differences between treatments for growth were not statistically tested since a potential tank effect on growth from tagging to 409 dph could not be excluded.

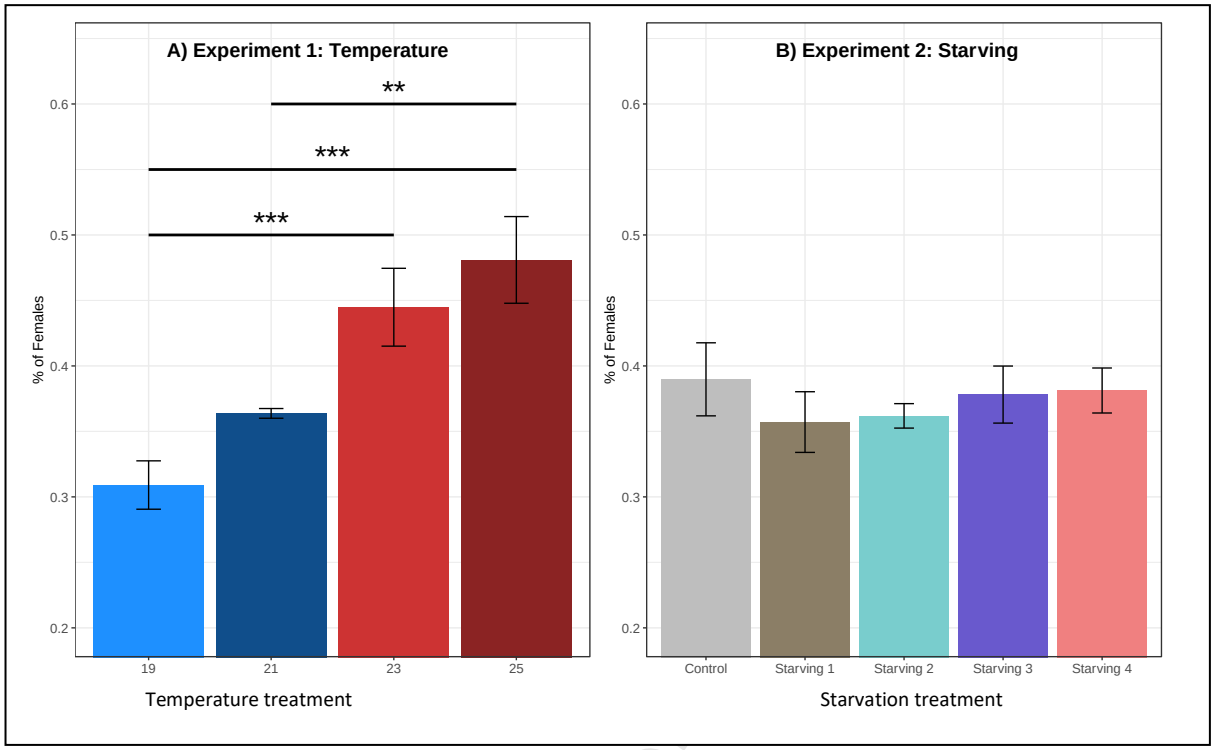

Figure 2 *Sex ratios (% of females) at sexing. A) Experiment 1: effects of four thermal treatments from 19 to 25 °C. B) Experiment 2: effects of four fasting periods from 60-65 dph to 123-133 dph. Significance levels for comparison of means: * P<0.05; ** P<0.01; *** P<0.001*

In Experiment 2, we observed an effect of each fasting treatment on body weight (Starving 1, F-value= 19.2, P<0.001; Starving 2, F-value= 18, P<0.001, Starving 3, F-value= 36.6, P<0.001, Starving 4, F-value= 6.4, P<0.05 - Table S2), indicating that fish indeed lost weight during fasting. However, the fasting period only significantly impacted length for the Starving 1 (F-value= 6, P<0.05) and Starving 3 (F-value= 11, P<0.01) treatments, compared to control (Table S3). We sexed a total of 4039 fish and there were no effects of the fasting treatment on the sex ratio of fish (F-value= 0.93, P>0.05; Figure 2B). There were also no differences at 390 dph for final weight (F-value= 0.75, P=0.6) and length (F-value= 0.93, P=0.5) among fasting treatments.

Discussion

Most previous studies dealing with the effect of temperature on sex determination in European seabass focused on the early phases of larval development (Piferrer et al. 2005; Mylonas et al. 2005). Mylonas et al (2003; 2005). They showed that the masculinizing effect of exposure to warm temperature was higher at the early larval stages. Similarly, the earliest the exposure to cold temperatures, the strongest the effects on feminization. Finally, a change in temperature from warm to cold (≈ 22 °C to 15 °C) at 57 day post fertilization did not produce any female (Blázquez et al. 1998), showing that 57 dph is too late to observe a feminizing effect of cold temperature. Our results highlight a clear effect of late

temperature (post 90 dph) on sex determination in the European seabass. Exposure to warm temperature (following a cold period at 16 °C from 0 to 90 dph) led to the production of more females. This is unusual since most of the literature on TSD in fish pinpoints that warm temperature promotes masculinization (Ospina-Alvarez & Piferrer, 2008, Geffroy and Wedekind, 2020). To our knowledge, examples of feminization by warm temperature are scarce in the fish literature. In the channel catfish (*Ictalurus punctatus*), thermal treatments during the labile period have shown a deviation of sex ratio in favor of females (63%) at 34 °C, *vs*. 50% and 55% at 27 and 20 °C, respectively (Patiño et al. 1996).

This brings new insights on recent findings indicating that exposure to low temperature during the juvenile stage increased the percentage of males (Vandeputte et al. 2020). Our results echo to those on the Atlantic silverside, *Menidia menidia*, a well-studied fish displaying temperature sex determination (Conover and Kynard, 1981). In this species, sex ratio is biased in favour of females at a relatively cold temperature, representative of the early breeding season, and male biased at a warmer temperature, representative of the late breeding season. The Atlantic silverside is a mass spawning species in which females are larger than males (Conover and Kynard, 1981), and in which fecundity and fitness are correlated to the size of the females, but not to that of males. While cold temperature induces a lower specific growth rate, which is *a priori* not beneficial, its benefit to females would be linked to cold being interpreted as a sign of earliness in the season, which is advantageous for females which then have more time to grow and become bigger for reproduction (Conover 1984). The European seabass shares some key features with *Menidia menidia*. It is a mass spawning species, females grow faster and are bigger than males, and their fitness is correlated with their size (Sadoul et al. 2022). We can assume that early cold temperature can similarly be a sign of earliness in the season, leading to a longer growing season and in the end larger size for potential females. Then, late warm temperatures might provide good condition for growth, which would favour females even more.

Recent studies on roach (*Rutilus rutilus*) and zebrafish (*Danio rerio*) demonstrated that sex differentiation was mediated by growth rate, with individuals with higher growth rate becoming females (Paull et al. 2009; Lawrence et al. 2008). However, in our study (experiment 2), we did not observe any effect of growth as fasting did affect growth but had no effect on sex determination. Such results suggest that late temperature might act on sex determination directly, and not through a growth-mediated process. Manipulation of growth through restricted feeding at much later stages (i.e., 130 to 400 dph) also did not affected sex ratio in European seabass (Diaz et al., 2013). Nonetheless, it might be that our fasting periods have been too short to induce an effect on sex, as they were in the end fully offset by compensatory growth, as shown by the similar body weight of all treatments at the end of Experiment 2. Indeed, food-related stress was likely too short, and we cannot preclude that a longer exposure to harsh conditions would have led to the expected effects.

Another likely process, linking temperature and sex, could involve DNA methylation of key genes involved in sex differentiation in fishes (reviewed in Piferrer et al. 2019). It has been shown that warm temperature causes epigenetic alteration of the promoter of the aromatase in the European seabass, resulting in masculinization (Navarro-Martin et al. 2011), while in barramundi (*Lates calcarifer*) low temperature resulted in male-specific DNA methylation (Budd et al., 2022). This methylation typically happens before the start of the thermal treatments we used here (90 dph), where there is no differentiation of gonads in European seabass, neither histologically, nor in terms of gene expression (Roblin and Bruslé 1983; Blázquez et al., 2009, Geffroy et al., 2021a; Saillant et al., 2003b). Differences in gene expression in the gonad between future males and females typically appear around 110-120 dph (Blázquez et al., 2009; Ribas et al., 2019; Geffroy et al., 2021a), so during our thermal treatment period. The histological differentiation of the gonad then starts after $1500DD_{10}$ (day.degrees with 10°C base), which corresponds to the end of the treatment in all groups. We do not know by which mechanism late warm temperature affects sex determination in sea bass, but owing to the timing it likely acts on the expression of key sex determining genes, potentialized by the epigenetic "female prone" absence of methylation of the aromatase promoter that the initial cold rearing period permits.

As highlighted before, we can hypothesize that the initial early cold temperature in our study mimicked the earliness in the breeding season, which is expected to be favourable to females. In such a framework, we also tested the potential effect of photoperiod by comparing a short photoperiod (10L:14D), representative of January-like conditions (early breeding season) versus a balanced one (12L:12D), representative of March-like conditions (late breeding season). No effect of photoperiod was detected. A previous attempt to test the effect of photoperiod on sex determination in European seabass was performed by Blasquez et al (1998), who did not find a significant effect of long photoperiod (15L:9D; 93% males) compared to short photoperiod (9L:15D; 97% males). However, in their case, the very high percentage of males due to early exposure to warm temperature (20-24 °C) may have masked a potential effect of photoperiod. In another Atherinopsidae with TSD, the pike silverside (*Chirostoma estor*), continuous light illumination from fertilization to the end of sex differentiation produced 73% of phenotypic males, compared to 40% at natural (12L:12D) photoperiod (Corona-Herrera et al., 2018). However, the authors suggested that such masculinization could be the result of a stress induced by continuous light exposure rather than a direct effect of photoperiod. Indeed, they showed that transcripts of the corticotropin releasing factor *crf* (a key regulator of the stress axis activation), were significantly upregulated following exposure to continuous illumination (Corona-Herrera, 2018). In that sense, cortisol, the major stress hormone in fishes, is a central transducer of environmentally-induced masculinization in several fish species (Hattori et al, 2009; Yamaguchi et al. 2010). For instance, pejerrey (*Odontesthes bonariensis*) larvae treated with cortisol at a temperature which normally leads to a balanced sex ratio (24 °C) show a strongly male sex ratio (100%). On the other hand, untreated larvae, submitted to a high temperature (29 °C) which produces 100% of males, had the highest cortisol

titre, while those larvae exposed to a low temperature (17 °C, which produces 100% females) presented the lowest levels of cortisol (Hattori et al. 2009). Regarding European seabass however, a recent study ruled out any potential effects of cortisol on sex determination and sex differentiation (Goikotxea et al. 2022). In the seabass, conditions of larval growth, rather than stress level, could indeed influence the sex ratio. In experiment 1, there was a clear effect of temperature treatments on growth, with the warmer treatments reaching the tagging size (8cm) earlier (160 dph for the 25 °C treatment, 192 dph for the 19°C treatment). In this case, a growth-mediated effect of temperature on sex cannot be ruled out. Recently, Geffroy et al. (2021) also demonstrated that breeding larvae of European seabass at low density (30 larvae per liter) which is favourable to growth, leads to significantly more females than at high density, conversely to Saillant et al. (2003a) who concluded that larval rearing density had no effect on the sex ratio. In addition, broodstock selected for growth also yield more females in their offspring than those from wild fish (Geffroy et al. 2021).

Conclusion

Although our experimental conditions did not permit us to demonstrate any effect of photoperiod nor of starvation on the sex ratio, without completely ruling out these possibilities, we showed that late exposure to warm temperatures (after 90 dph), after an initial cold treatment, does modulate sex ratio in European seabass, with warm temperatures (23-25 °C) favouring female differentiation. Practically, we recommend that farmers interested in producing females breed larvae of *Dicentrarchus labrax* first at a cold temperature (<17 °C) until 90 dph, and then increase that temperature up to 23-25 °C until 160 dph. That, combined with selection for growth, should be a good way to produce a high proportion of females in farmed populations of European sea bass.

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights revised

- Late high temperature can yield females in seabass.
- This can be applied to improve seabass production
- We found no effect of photoperiod or starvation on sex ratio.