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BRIEF COMMUNICATION

Effects of daily thermal fluctuations on the Atlantic silverside, a fish with temperature-dependent sex determination

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Abstract

Temperature-dependent sex determination (TSD) occurs when the temperature during development affects gonad determination. Historically, most work on TSD in fishes was conducted under constant temperatures, yet daily fluctuating temperatures can significantly alter fish physiology and life history. Thus, we subjected the Atlantic silverside, *Menidia menidia* (a TSD species), to 28, 28 ± 2 and 28 ± 4°C (a high, masculinizing temperature) and quantified sex ratios and length. We found that the percentage of females increased by 60%–70% when the fish were exposed to daily fluctuating temperatures (from 10% to 16% and 17% under fluctuations).

KEYWORDS
fluctuations, *Menidia*, temperature sex determination

1 | INTRODUCTION

In many fishes, the sex of an organism can be influenced by the temperature of the environment (Ospina-Alvarez & Piferrer, 2008). This phenomenon, known as temperature-dependent sex determination (TSD), typically involves a thermosensitive period early in life during which the development of gonads is affected by temperature (Georges *et al*. 2004). In the Atlantic silverside (*Menidia menidia*), an atheriniform estuarine fish with an august history of TSD research (Conover & Heins, 1987a; Conover & Kynard, 1981), this period is based on size: it occurs when larvae are between 8 and 21 mm, with colder temperatures leading to an overproduction of females and *vice versa* (Conover & Fleisher, 1986).

As is common in thermal physiology studies (Sheldon & Dillon, 2016), however, most work on TSD in fishes has been conducted under constant temperatures (Bowden & Paitz, 2018). In these experiments, larvae spend their entire thermosensitive period at one temperature. While these experiments were vital in establishing the nature of this phenomenon, they failed to account for the fluctuating nature of temperature in the wild. We now know that organisms under constant thermal regimes are quite different to those exposed to daily varying temperatures in terms of physiology and life history (Carrington *et al*., 2013; Carroll & Quiring, 1993; *e.g*., Morash *et al*., 2018; Salachan & Sørensen, 2017; Salinas *et al*., 2019). It is thus possible that daily fluctuations could change the dynamics of TSD. In turtles and squamates, that is precisely what has been found (Georges *et al*., 1994; Harlow & Shine, 1999; Paitz *et al*., 2010; Valenzuela *et al*., 2019). For example, northern painted turtle clutches go from 0% females at 27°C to 30% at 27 ± 4°C to 100% at 27 ± 8°C (Paitz *et al*., 2010). However, this the issue remains largely unexplored in fishes. Honeycutt *et al*. (2019) observed a very slight difference in sex ratios when growing southern flounder at constant 27°C and at a naturalistic fluctuating temperature that averaged 27°C (98 *vs*. 100% males).

How daily thermal fluctuations affect sex ratios is a particularly timely area of study as water temperature variability continues to increase (Wang & Dillon, 2014). Species with TSD could be severely impacted by increased thermal variability, resulting in skewed sex ratios and population declines (Hawkes *et al*., 2007; Janzen, 1994; Wedekind, 2017). To address this, we quantified sex ratios, as well as growth, under a constant temperature of 28°C, a masculinizing condition (Duffy *et al*. 2015), and two levels of daily fluctuating thermal conditions (28 ± 2 and 28 ± 4°C) in *M. menidia*. We hypothesized that fluctuations would result in more females (*i.e*., a less male-heavy sex ratio) and smaller larvae, as found in many other fishes (*e.g*., Hirakawa & Salinas, 2020).

2 | MATERIALS AND METHODS

2.1 | Collection and rearing

We used *M. menidia* from Charleston, South Carolina, USA. Ripe adults were captured with a seine net in Charleston Harbour's shallow waters during two different periods in 2021: 11–12 May (batch 1) and 28 May to 1 June (batch 2). Fish were brought into the Grice Marine Laboratory and strip-spawned after a 1-day acclimation period, following protocols approved by the College's Institutional Animal Care and Use Committee (IACUC ID 2021–005). Fertilized eggs were transferred to an iodine bath (polyvinylpyrrolidone-iodine1) for 15 min to prevent fungal growth and then shipped overnight to Kalamazoo College.

The experiments were conducted in the fish-rearing facility at Kalamazoo College and all protocols were approved by the College's Institutional Animal Care and Use Committee. We used 18 56 L tanks (155 × 70 × 25.5 cm), each independently maintained with an EHEIM 2217 canister filter (EHEIM GmbH, Deizisau, Germany) and an Apex Jr. (Neptune Systems Inc., Morgan Hill, CA) controller that monitored and adjusted temperature continuously. These large tanks contained many smaller growth chambers with holes covered by fine mesh, which allowed for water flow. Standard rearing conditions were maintained throughout [20 practical salinity units, a salinity typically used to raise silversides with no effect on sex ratios (Conover & Heins, 1987a); daily monitoring of ammonia, nitrite and nitrate; weekly water changes; photoperiod of 16 h:8 h light:dark to simulate summer conditions].

We fed fish twice a day *ad libitum*. Each feeding consisted of 1-day-old brine shrimp and Otohime dry fish food (A1or B2 depending on larval size; Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan). As embryos and larvae, the fish were housed in 13.4 × 17.3 × 13.2 cm Marina Fine Mesh Net Breeders (hagen Inc, baie d'Urfé, QC, Canada). They were transferred to larger 40.6 × 33.3 × 25.4 cm growth chambers once they were ~15 mm. During this transfer, we aimed to standardize fish density per chamber to 14 individuals (range = 13–17, mean = 13.94, S.D. = 0.8). When they reached ~15 mm, we also started introducing small amounts of frozen adult brine shrimp in addition to the Otohime dry food.

2.2 | Temperature treatments

Three different temperature treatments were used: 28°C as the constant temperature, a daily fluctuation treatment of 28 ± 2°C (26°C at the lowest point and 30°C at the highest, low variability) and one of 28 ± 4°C (24–32°C, high variability). The temperature treatments were chosen based on the thermal profile of Charleston Harbour as well as that of a nearby site (Appendix 1). Water temperature was controlled and maintained with tank-specific Apex Jr. controllers, and the three thermal environments were replicated six times (*i.e.*, six tanks of each treatment for a total of 18 tanks). Each tank housed four large growth chambers with ~14 fish in each (consequently, each treatment had ~335 fish, with a total fish count in the experiment of 964 individuals). Logistical constraints prevented us from replicating these treatments under low-temperature, feminizing conditions.

The two different shipments of embryos were treated slightly differently. The first (batch 1, ~800 embryos from 11 pairs) was subdivided into the three temperature treatments after 1 day of acclimation to our fish-rearing facility (still in the embryo stage) at 28°C. The other (batch 2, ~1000 embryos from 28 pairs) was kept at 28°C on receipt and reared at this constant temperature until they were 7 days old (for a schematic of the methodology, see Appendix 2). This second protocol has been used in previous *M. menidia* experiments (*e.g.*, Conover & Heins, 1987b).

We photographed the larvae before transferring them to the different treatments and ascertained that they had not entered their thermosensitive period. A subset of fish from batch 2 was measured at 6 days old, before the transfer, by photographing the fish in ~1 cm of water. The images were then calibrated and measured using ImageJ (Schneider et al. 2012). The mean length of these fish was 4.54 mm (S.D. = 0.866, n = 141). *M. menidia*'s thermosensitive period was documented to be between 8 and 21 mm (Conover & Fleisher, 1986).

2.3 | Measurements

All fish were euthanized with MS-222 and their total length recorded with callipers. We then sexed them under a dissection scope by visual identification of gonads. Photographs from the gonads of 22 random individuals were sexed by G.M.H. and Dr. Tara Duffy (Northeastern University, an expert on *M. menidia* sex identification) independently. After obtaining 100% correspondence (the sample included both sexes), all remaining individuals were sexed by G.M.H. alone.

2.4 | Statistics

We analysed sex ratios *via* a generalized linear model with a binomial error distribution and a logit link function. Sex was used as the response variable and temperature treatment and batch number [*i.e.*, whether individuals were placed into temperature treatments as embryos (batch 1) or as larvae (batch 2)] as the independent ones. We included batch number in the statistical model to evaluate whether the two different early-life protocols influenced the sex ratio results. For length-at-age, we used a generalized linear model with a Gaussian error distribution and identity link function.

3 | RESULTS

3.1 | Sex ratio

Both temperature treatment and batch number influenced the sex ratio of fish (generalized linear model: temperature $P = 0.021$, batch number $P = 0.002$) but their interaction did not (generalized linear model: $P = 0.749$). In the high variability treatment (28 ± 4°C), 17% of the fish were females, a significantly higher proportion than the 10% of females under constant 28°C ($z = -2.01$, $P = 0.044$; Table 1). The

TABLE 1 Sex ratios of fish exposed to constant 28°C and the two daily fluctuating treatments for each separate batch (batch 1: individual fish placed at their assigned temperature treatment as embryos; batch 2: larvae moved to their assigned treatment ~6 days post-hatch after spending the beginning of their lives at constant 28°C)

Treatment	% Female	*N*
28°C batch 1	5%	95
28°C batch 2	11%	226
28°C total	**10%**	**321**
28 ± 2°C batch 1	12%	222
28 ± 2°C batch 2	18%	114
28 ± 2°C total	**16%**	**336**
28 ± 4 °C batch 1	11%	193
28 ± 4°C batch 2	22%	102
28 ± 4°C total	**17%**	**295**

Abbreviation: *N*, number of fish in each treatment.

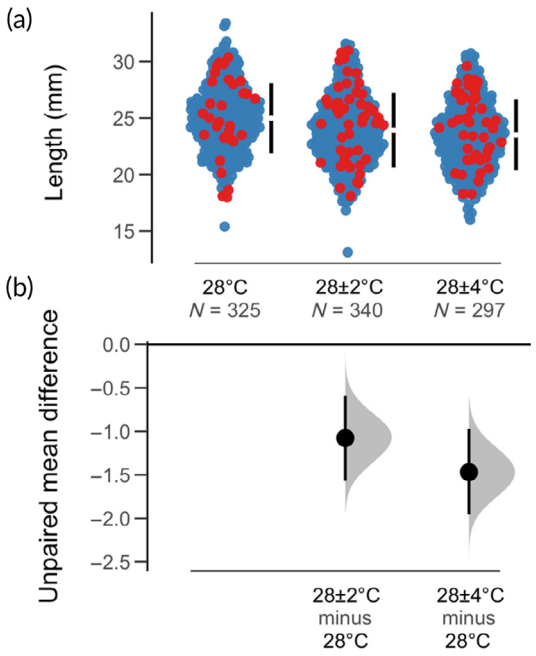

FIGURE 1 Cumming plot showing: (a) the mean difference in total-length-at-age between each temperature regime, each point representing an individual fish (blue = male, red = female) and (b) the corresponding mean difference in each fluctuating treatment along with a bootstrap sampling distribution. The black dots indicate mean difference and the vertical lines represent 95% confidence intervals. ●, female; ●, male.

difference between the sex ratios in the low variability (28 ± 2°C) and constant treatments was borderline significant ($z = -1.96$, $P = 0.050$; Table 1). There was no difference between sex ratios at the two fluctuating treatments ($z = -0.1037$, $P = 0.920$; Table 1).

3.2 | Length-at-age

Fish reared at a constant temperature were significantly longer than those at either of the two fluctuating temperature treatments (generalized linear model: $P < 0.001$; Figure 1a and Appendix 3). There was no significant difference between the length of the fish in the two fluctuating temperature treatments or between the two sexes (Figure 1a and Appendix 3).

4 | DISCUSSION

Somewhat surprisingly, we found that the previously observed effect of high temperature, in which TSD skews the sex ratio towards males, was less pronounced when temperatures were allowed to fluctuate daily. The timing of exposure to diel fluctuations also mattered: larvae who spent 7 days under constant 28°C before fluctuations consistently produced more females regardless of treatment, including under constant 28°C. This paradoxical result may be explained by the existence of a genetic component to each individual *M. menidia*'s response to temperature during sex determination (Conover *et al.*, 1992; Conover & Heins, 1987b; Conover & Kynard, 1981). Since our embryos came from two different sets of parents, the difference could be a result of genetic differences. Despite this difference between batches, more females were produced under diel fluctuations—at similar rates—in batches 1 and 2. Length at the end of the experiment was significantly greater in fish grown at the constant temperature. This finding is common in ectotherms, can be explained by Jensen's inequality (Denny, 2017) and should be incorporated into models hoping to predict responses to climate change.

Our results at constant 28°C are in line with previous findings in nearby populations. Duffy *et al.* (2015) observed 12% females in a population at 32.7°N and 3% in another at 33.4°N (ours was at 32.7°N). Conover & Heins (1987) and Conover *et al.* (1992) reported percentage female values of ~20 and 17, although in both those studies fertilized embryos were collected from the wild and brought into the laboratory (Duffy *et al.*) and we strip-spawned adults. Regardless, we acknowledge that the fish we sexed were small and the possibility of mis-sexing exists (no genetic sex markers have been found in *M. menidia*, which is why we had to rely on morphological observations).

A speculative, but intriguing, possibility is that the differences in sex ratio could be linked to stress. Cortisol was recently shown to masculinize *Odontesthes bonariensis*, a South American silverside with TSD (Strüssmann *et al.*, 2021). When released, this hormone increases the expression of *amh* and decreases that of *cyp19a1a*, two genes important in sex determination, resulting in gonadal apoptosis and a higher proportion of males at an intermediate, sexually-neutral temperature (Hattori *et al.*, 2009). Although it was first described in response to thermal stress, other types of conditions that ramp up cortisol production were also found to affect sex ratios (Hattori *et al.*, 2020). Our results could then be explained if daily fluctuating temperatures impact cortisol production. Fewer males at our

fluctuation treatments would suggest that these fish had lower cortisol levels than their constant 28°C counterparts. Evidence for stress responses under fluctuations is mixed: in some cases, cortisol is ramped up during diel thermal variation (Takahara *et al*., 2011), but in others, cortisol is elevated under constant conditions (Davis *et al*., 2001). The choice of temperature treatments is likely quite important: a constant temperature near a population's optimal temperature would produce different results to choosing one close to the upper thermal limit (Hokanson *et al*., 1977), as predicted by Jensen's inequality (Rule & Ayres, 1999). More work is certainly needed to establish the link between cortisol, temperature fluctuations and sex ratios.

TSD under thermal variability is a pressing issue: the range of diurnal temperature cycling over the period 1975–2013 has increased in temperate areas by 0.96 ± 0.12°C, a larger increase than that of mean annual temperature (Wang & Dillon, 2014), therefore accurately predicting sex ratio responses in species with TSD is critical. It will also be more difficult than previously thought given our finding that the sex determination process is influenced by more realistic conditions. To be fair, the resulting sex ratios are not wildly different to the constant-temperature derived ones, but it is important to realize that small differences could have a significant impact on population dynamics estimates (Geffroy & Wedekind, 2020; Wedekind, 2017).

AUTHOR CONTRIBUTIONS

Santiago Salinas: conceptualization, methodology, analysis, resources, writing – original draft, writing – review and editing. Grace M. Hancock: conceptualization, methodology, investigation, writing – original draft, writing – review and editing. Stephan B. Munch: analysis, writing – review and editing. Gorka Sancho: resources, writing – review and editing.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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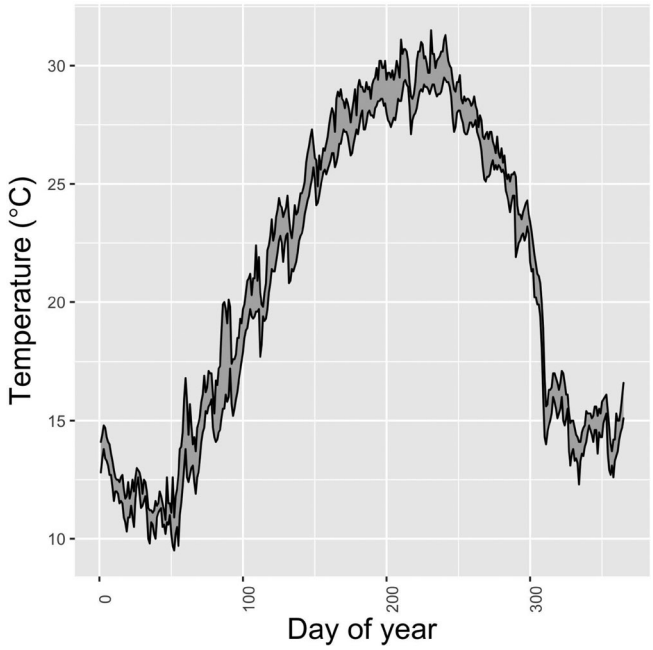
APPENDIX 1: Daily temperature variation over 2021 recorded at the Fort Johnson station ("ACEFJWQ", Ace Basin National Estuarine Research Reserve), a site near our collection site at Charleston Harbour



APPENDIX 3: Multiple comparison tests after Bonferroni correction for length-at-age for each temperature treatment. Fish reared under constant 28°C were longer than those under both fluctuating conditions.

Treatment	*vs*	Mean difference	Standard error	*P*
28°C treatment	28 ± 2°C treat	1.076	0.246	0.000
	28 ± 4°C treat	1.469	0.254	0.000
28 ± 2°C treat.	28°C treatment	−1.076	0.246	0.000
	28 ± 4°C treat	0.392	0.252	0.361
28 ± 4°C treat.	28°C treatment	−1.469	0.254	0.000
	28 ± 2°C treat	−0.392	0.252	0.361

APPENDIX 2: Schematic representation of the protocol followed for batches 1 and 2