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Original Article

Temperature-dependent effects on fecundity in a serial broadcast spawning fish after whole-life high CO_2 exposure

Callie A. Concannon[1], Emma L. Cross[2], Lucas F. Jones[1], Christopher S. Murray[3], Catherine M. Matassa[1], Richard S. McBride[4], and Hannes Baumann [1,*]

[1]*Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, CT, 06340, USA*
[2]*Department of the Environment, Geography, and Marine Sciences, Southern Connecticut State University, 501 Crescent Street, New Haven, CT, 06515, USA*
[3]*Washington Ocean Acidification Center, School of Marine and Environmental Affairs, University of Washington, G 3707 Brooklyn Ave NE, Seattle, WA, 98105, USA*
[4]*National Marine Fisheries Service, Northeast Fisheries Science Center, 166 Water Street, Woods Hole, MA, 02543, USA*

[*]*Corresponding author: tel: 860-405-9297; e-mail: hannes.baumann@uconn.edu*

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Experiments examining fish sensitivities to future oceanic CO_2 levels have greatly expanded over past decades and identified many potentially affected traits. Curiously, data on reproductive trait responses to high CO_2 are still scarce, despite their strong link to Darwinian fitness and thus to population vulnerability to ocean acidification. We conducted two rearing experiments on the first broadcast-spawning marine fish model (Atlantic silverside, *Menidia menidia*) to examine how long-term and novel whole life-cycle exposures to predicted future CO_2 levels (~2,000 μatm) affect laboratory spawning, temperature-specific reproductive investment, fecundity, and size distributions of maturing oocytes. At low temperatures (17°C), female body size and therefore potential fecundity (F_{Pot}, oocytes/female) slightly increased with CO_2, while relative fecundity (F_{Rel}, oocytes/g female) remained unaffected. At high temperatures (24°C), high CO_2 substantially reduced both F_{Pot} (−19%) and F_{Rel} (−28%) relative to control treatments. Irrespective of CO_2, females at 24°C grew larger and heavier than those at 17°C, and although larger females produced larger oocytes at some developmental stages, they also had lower gonadosomatic indices and lower F_{Rel}. Our findings contrast with most previous studies and thus highlight the need to investigate reproductive impacts of increasing CO_2 on multiple fish species with contrasting life history strategies.

Keywords: asynchronous batch spawner, Atlantic silverside (*Menidia menidia*), fitness, ocean acidification, temperature.

Introduction

The threat of unfolding marine climate change has motivated rapidly expanding research on the CO_2 sensitivity of marine organisms in recent decades (Boyd *et al.*, 2018; Doney *et al.*, 2020). Most studies have used experimental approaches to reveal important, first-order insights into species and traits affected by predicted future CO_2 levels and into mechanisms causing such effects (Baumann, 2019). The rich amount of empirical data now contains a complex spectrum of negative, neutral, and positive responses to acidification within as well as across traits and species (Kroeker *et al.*, 2013; Przeslawski *et al.*, 2015), which cumulatively suggest a shifting marine fitness landscape but still limit more specific predictions. Experiments on marine fishes have shown that short-term high CO_2 exposures can reduce survival in some species, but also that such effects are generally confined to the earliest, least developed life stages (Chambers *et al.*, 2014; Baumann *et al.*, 2018; Cattano *et al.*, 2018; Murray *et al.*, 2019). Most other documented responses to high CO_2 in fishes comprise non-lethal changes to e.g. metabolism (Munday *et al.*, 2009), growth (Murray and Baumann,

2020), behaviour (Ashur *et al.*, 2017), or otolith formation (Bignami *et al.*, 2013), which arise as by-products of efficient acid-base regulation and swift CO_2 acclimation (Esbaugh, 2018). While potentially important, the challenge for many of these CO_2 responses often lies in drawing unequivocal links to Darwinian fitness, which is what matters most, because it measures the success of an individual in contributing to the next generation.

Curiously, very few studies to date have examined fecundity or reproductive output in fishes under future CO_2 environments, even though reproductive traits are closely linked to Darwinian fitness and thus to overall species vulnerabilities to ocean acidification (Nagelkerken *et al.*, 2021). Such experiments are clearly more complex, require longer durations, or face challenges in identifying appropriate fish models. However, their scarcity has left a knowledge gap preventing better assessments of ocean acidification as an emerging threat for marine fishes. Pioneering work has exclusively tested fish species with parental care, where experimenters could select adults or sub-adults from the wild to then quantify frequency and sizes of egg clutches laid by breeding pairs under contrasting CO_2 conditions (Miller *et al.*, 2013; Welch and Munday, 2016; Faria *et al.*, 2018). No comparable data exist yet for broadcast spawners, a group that includes most commercial fishes. Moreover, no marine fish model has yet been reared from fertilization to maturity to examine how realistic whole-life CO_2 exposures may affect reproductive output.

To address this knowledge gap, we conducted two long-term rearing experiments on the Atlantic silverside (*Menidia menidia*, hereafter: silverside), a broadcast-spawning forage fish along the North-American Atlantic coast (Middaugh *et al.*, 1987) and well-studied model in evolutionary, ecological, and climate change research (e.g. Conover and Kynard, 1981; Billerbeck *et al.*, 2000; Conover *et al.*, 2009; Cross *et al.*, 2019). Silversides are particularly amenable to whole-life studies because of their relatively short, annual life span and the existence of established laboratory protocols for whole-life rearing (Conover and Munch, 2002). Furthermore, silversides affix fertilized eggs to submersed vegetation in the wild (or to artificial substrates in the laboratory), which facilitates offspring collection and quantification. However, known challenges to whole-life rearing include their obligate schooling behaviour and batch spawning, which demand large rearing tanks (>500 L) and associated space-replication trade-offs. We therefore used two complementary approaches over two experimental seasons to quantify reproductive output under contrasting CO_2 environments in silversides. The first approach (hereafter: *spawning trial*) reared wild-caught early juveniles to maturation and then quantified embryo production in laboratory populations over a 2-month period. The second approach (hereafter: *fecundity trial*) reared silversides from fertilization to just prior to spawning, in order to quantify individual female reproductive investment, fecundity, and oocyte development via macroscopic and histological analyses. We hypothesized that long-term or whole-life exposures to future CO_2 conditions would have small but accruing metabolic costs, which would result in lower reproductive investment, lower fecundity, and/or smaller oocytes in silversides reared at high compared to control CO_2 conditions.

Material and methods

Experimental CO_2 and temperature treatments

The spawning trial reared wild-caught juveniles to maturity at 20°C under duplicate control (∼600 µatm CO_2) and high CO_2 conditions (∼2200 µatm CO_2). The fecundity trial reared newly fertilized embryos to maturity under control vs. high CO_2 conditions and two temperatures (17°C: ∼500, ∼2000 µatm CO_2; 24°C: ∼500, ∼2600 µatm CO_2; Table 1). High CO_2 conditions were chosen to approximate the predicted surface ocean maximum in coming centuries (Caldeira and Wickett, 2003) that is a common benchmark in ocean acidification studies (Riebesell *et al.*, 2011). However, comparably high CO_2 levels already occur seasonally (late summer–fall) in nearshore silverside habitats (Baumann *et al.*, 2015) and may become more frequent and extreme in future decades (Shaw *et al.*, 2013; McNeil and Sasse, 2016). Temperature treatments were consistent with past experimental work, representing average thermal conditions at the beginning (17°C), height (20°C), and end (24°C) of the species' spawning season in Long Island Sound (Baumann *et al.*, 2015; Murray and Baumann, 2018), with 24°C being the thermal optimum for larval and juvenile growth (Middaugh *et al.*, 1987).

Experimental treatment tanks were continuously bubbled with mixes of air and 100% bone-dry CO_2 controlled by gas proportioners (ColeParmer®). In control CO_2 treatments, metabolic acidification by the fish was offset by bubbling CO_2-stripped air (∼50 µatm CO_2) via diffuser tubes into the rearing tanks (Murray and Baumann, 2020). All treatments maintained near 100% dissolved oxygen saturations (∼8.1, 7.6, and 7.1 mg L^{-1} at 17°C, 20°C, and 24°C, respectively). Salinity was monitored daily via refractometer and adjusted as necessary to maintain a target of 31 psu. Temperature conditions were maintained by thermostats (Aqualogic®) connected to submersible heaters or in-line chillers (DeltaStar®), and target pH conditions were monitored daily using a handheld pH meter (Hach® Intellical PHC281 pH electrode with HQ11D handheld pH/ORP meter, calibrated bi-weekly using two-point NIST buffers). Achieved pCO_2 levels were calculated via CO2SYS (v2.1) from total alkalinity (A_T) measurements in samples of tank and lab source water ($N_{Spawn} = 12$, $N_{Fecund} = 17$) measured via endpoint titration (G20 Potentiometric Titrator, Mettler Toledo®) with an accuracy of ± 1% (Dr Andrew Dickson's certified A_T reference, batch number 162). Using A_T, pH, temperature, salinity, and K2 constants from Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987) and Dickson (1990), we derived CO_2 partial pressure (pCO_2, μatm), dissolved inorganic carbon (C_T, μmol kg^{-1}), and carbonate ion concentrations (CO_3^{2-}, μmol kg^{-1}) given in Table 1.

Offspring production and laboratory rearing

For the spawning trial, juveniles were caught on 25 September 2015 via beach seine (30 × 2 m) in Mumford Cove, a small seagrass-dominated embayment connected to eastern Long Island Sound (41.32°N, 72.02°W). Individuals were transported to the Rankin Seawater Facility at the University of Connecticut (Groton, CT, USA). A total of 100 individuals (mean total length [TL] = 4.5 cm) were randomly assigned to each of two 700-L replicate rearing tanks per CO_2 treatment and reared to maturity over the next five months at 20°C ($N_{total} = 400$). Maturation was achieved via photoperiod manipulation, i.e. by reducing daylight from 15 h to 12 h during months 3–4 followed by a reversal back to 15 h for the final (fifth) month (Conover and Munch, 2002; Salinas *et al.*, 2012). Fish were fed equal amounts of pelleted food (Otohime Marine Fish Diet, size B1, B2, C1, Reed Mariculture®), delivered continuously to each tank during light hours via belt feeders.

For the fecundity trial, spawning-capable silversides were captured in Mumford Cove on 29 May 2018, transported to the laboratory, separated by sex, and held overnight without food in

Table 1. Overview of experimental water chemistry during the spawning and fecundity trials, using measured temperature (°C), pH (NIST), and total alkalinity (A_t, μmol kg^{-1}) to calculate achieved mean ± *SD* of pCO_2 (μatm), dissolved inorganic carbon (C_t, μmol kg^{-1}), and carbonate ion concentrations (CO_3^{2-}, μmol kg^{-1}).

	CO_2	Temp	pH	A_t	pCO_2	C_t	CO_3^{2-}
Spawning trial	Control CO_2	20 ± 0.5	7.99 ± 0.08	2 035 ± 74	614 ± 147	1 893 ± 90	108 ± 12.2
			7.98 ± 0.04	2 044 ± 49	620 ± 87	1 905 ± 59	107 ± 5.9
	High CO_2		7.46 ± 0.02	2 030 ± 57	2 252 ± 161	2 050 ± 60	35 ± 1.0
			7.47 ± 0.04	2 054 ± 70	2 217 ± 295	2 071 ± 80	37 ± 1.2
Fecundity trial	Control CO_2	16.9 ± 0.6	8.07 ± 0.11	2 023 ± 9	489 ± 136	1 869 ± 44	115 ± 26
		23.7 ± 1.2	8.06 ± 0.13		538 ± 178	1 839 ± 58	135 ± 35
	High CO_2	16.9 ± 0.9	7.50 ± 0.09		2 024 ± 445	2 045 ± 25	34 ± 6
		23.6 ± 1.2	7.42 ± 0.12		2 628 ± 882	2 048 ± 39	36 ± 9

80-L aerated containers at ambient CO_2 and temperature conditions (17°C). The following day, 20 females and 27 males were strip-spawned together using established protocols (Baumann *et al.*, 2018; Murray and Baumann, 2020). Within 2 hours post fertilization, screens with attached embryos were randomly placed into 32 rearing containers (20-L, with flow-through screening), which were then distributed and placed within the four 700-L rearing tanks (n = eight containers/tank). Newly hatched larvae were fed *ad libitum* amounts of brine shrimp nauplii (*Artemia salina*, San Francisco strain, brineshrimpdirect.com) augmented initially by a powdered weaning diet (Otohime Marine Fish Diet, size A1).

After reaching approximately 30 mm TL (45 days post hatch, dph, for 24°C, 73 dph for 17°C), fish were counted, measured (n = 10), and divided randomly among three 80-L rearing containers (with flow-through screening) placed back into their original 700-L tank and corresponding CO_2 and temperature treatment. At this point, each of the two 24°C tanks contained 148 juveniles, while each of the two 17°C tanks contained 96 individuals. At 104 dph (17°C) and 111 dph (24°C), juveniles were counted and measured again then placed directly into their original 700-L tank and corresponding treatment. Fish were fed daily rations of pelleted food via belt feeders (Otohime Marine Fish Diet, size B1, B2, C1) corresponding to 20% of their estimated dry weight biomass per tank (dW_{all}). We readjusted dW_{all} daily using published temperature-specific TL growth data for silversides (Billerbeck *et al.*, 2000) and the empirical relationship $dW = 0.0012{*}TL^{2.997}$ (Murray *et al.*, 2017), multiplied by the number of fish in each tank. To avoid overcrowding as fish grew, at 245 dph half of the fish in each of the two 24°C tanks were randomly selected and moved into two new tanks receiving the corresponding temperature (24°C) and CO_2 treatments. Biological filtration (four-stage canister biofilters with 9-watt ultraviolet [UV] sterilizers, Polar Aurora®), regular solid waste removal, and weekly water changes with filtered (1 μm) and UV-sterilized seawater from Long Island Sound ensured undetectable ammonia concentrations throughout the experiment (<0.25 ppm, tested 3 × weekly; Saltwater Master Test Kit, API®). A photoperiod of 15 h light, 9 h dark was maintained throughout the embryo, larval, and juvenile stages. To induce maturation, the photoperiod was reduced to 12 h light for 2 months beginning 245 dph, followed by reversal back to 15 h light until sampling. The fecundity trial was terminated before spawning occurred at 317–325 dph, when all fish were euthanized with MS-222 (Tricaine-S).

Trait measurements

For the spawning trial, spawning in each tank began when silversides were provided bundled threads of green yarn suspended mid-tank. Yarns were checked every 48 h for attached embryos (i.e. water hardened eggs), which were counted and subsampled (n = 15) to measure egg diameters via calibrated pictures (4 × magnification, ImagePro Premier, v9.0, Media Cybernetics®). The spawning trial was terminated after 60 days, which is approximately the extent of silverside spawning season in the wild (Pringle and Baumann, 2019). Surviving fish were euthanized, measured for TL (0.1 mm) and wet weight (wW, 0.01g), and their sex determined by gonad inspection under a stereo-microscope (Nikon SMZ-1000). The total number of embryos spawned was scaled to the cumulative wet weight of females in each tank to obtain a mass-specific estimate of embryo production (n_{emb}/g female) per replicate tank. This necessarily assumed that all females within a tank participated in spawning.

For the fecundity trial, euthanized fish were measured for TL and wW, followed by dissection for sex identification and gonad removal. Re-weighing yielded the gonad-free body weight ($wW_{no\ gonad}$), which was subtracted from wW to yield gonad weight (wW_{gonad}) and the gonado-somatic index ($GSI = 100{*}wW_{gonad}/wW_{no\ gonad}$). Male gonads were discarded. Female gonads were preserved in 5% buffered formaldehyde/freshwater solution. One ovary lobe per female was used to quantify all secondary growth (i.e. vitellogenic) oocytes; the other was saved for histological analyses. To characterize secondary growth oocytes, the ovary lobe was divided into anterior, middle, and posterior sections that were each gently teased apart so that oocytes were evenly distributed among four rectangular 2 mL wells. Each well was photographed (3 × magnification) and analyzed via ImageJ (v1.52a, National Institute of Heath) with the ObjectJ plugin (v1.04t, University of Amsterdam) and a customized macro to count and measure the diameter of all oocytes. The total number of oocytes counted per lobe was doubled to obtain each female's potential fecundity (F_{Pot}, oocytes/female) and relative fecundity ($F_{Rel} = F_{Pot}$/wW, oocytes/g female). In addition, we averaged the size of the 25 largest oocytes per female (~99% percentile) as a measure of maximum oocyte size.

The other ovary lobe from a subset of 17 females at control CO_2 treatments ($n_{17°C} = 6$, $n_{24°C} = 11$) and 20 females at high CO_2 treatments ($n_{17°C} = 8$, $n_{24°C} = 12$) were embedded in paraffin, sectioned, placed on glass slides, and stained with Hematoxylin & Eosin (H&E) (Horus Scientific, Worcester, MA). Slides were then

Table 2. *Menidia menidia*: mean ± *SD* of response traits during the spawning trial (2015–2016) and the fecundity trial (2018–2019).

	Spawning trial		Fecundity trial			
CO_2 treatment	**Control**	**High**	**Control**	**High**	**Control**	**High**
Temperature (°C)	**20**		**17**		**24**	
$N_{females}$	53	65	40	17	11	17
TL (mm)	119.3 ± 9.9	115.5 ± 9.4	88.6 ± 8.2	94.2 ± 9.1	111.4 ± 6.7	115.0 ± 5.8
wW (g)	9.3 ± 2.1	8.7 ± 1.8	4.2 ± 1.1	5.4 ± 1.3	8.1 ± 1.4	9.1 ± 1.4
$N_{embryos}$/fem	550, 541*	174, 571*				
$N_{embyros}$/g fem	0.8, 2.5*	2.3, 2.3*	Not assessed			
Embryo diameter (μm)	1 131 ± 54	1 126 ± 50				
GSI (%)			18.9 ± 8.1	16.1 ± 5.4	8.1 ± 3.5	8.2 ± 1.8
Potential fecundity (oocytes/fem)			3 262 ± 1 348	4 355 ± 1 338	3 260 ± 747	2 631 ± 675
Relative fecundity (oocytes/g fem)			762 ± 221	807 ± 164	404 ± 53	291 ± 66
Maximum oocyte diameter (μm)	Not assessed		1 152 ± 139	1 113 ± 156	1 047 ± 219	917 ± 136
Median oocyte diameter (PG, CA, V1, V2, NM, H in μm)**			132, 289, 397, 577, 648, 804		135, 326, 435, 577, 601, 932	

*values given for each duplicate;
**values pooled across CO_2 treatments

photographed (4 × magnification) and six oocyte developmental stages identified: primary growth (PG), cortical alveolar (CA), early vitellogenesis (V1), late vitellogenesis (V2), nuclear migration (NM), and hydrating (H) oocytes (following Ganias *et al.*, 2004; Hyle *et al.*, 2014; Press *et al.*, 2014). We also checked for but did not find postovulatory follicles that would have indicated the beginning of spawning. Furthermore, we examined all histological images for the occurrence of alpha or beta atresia, following McElroy *et al.* (2016). Given that the incidence of atresia was not significantly different from zero (%) and also not significantly different between treatments, no adjustment to whole oocyte counts was necessary (see electronic Supplementary Material). Up to 56 oocytes per developmental stage per female were measured (mean = 13.5). Histological preparation deformed most oocytes to ellipses; therefore, we first calculated the area of each ellipse from its major and minor axis and then inferred the oocyte diameter (= size) from a circle with the same area. For each female, we determined the stage-specific median and maximum oocyte size, which were then compared across treatments.

Data analysis

Rearing large numbers of schooling fish to maturity is space demanding and, in a facility like ours, demanded experimental design trade-offs with respect to replication. With the replication unit being each 700-L tank (instead of each female), the spawning trial thus had duplicates for each CO_2 level (single temperature), while the fecundity trial had single replicates for each CO_2 × temperature combination. Although the 24°C treatments ended with two equal density groups per CO_2 level, these were not true replicates, because fish were only separated after 245 dph. Given that these duplicates were not significantly different from one another with respect to mean TL and wW (t-tests, both $p > 0.21$), they were pooled for all subsequent analyses. Lacking the treatment-level replication and normal distributions required by parametric statistical approaches (General Linear Model (GLM) and analysis of variance), we instead compared trait *distributions* between CO_2 and temperature treatments using non-parametric Mann–Whitney U tests (reporting *Z*-scores) with adjusted *p*-values for multiple comparisons during each trial (Benjamini and Hochberg, 1995). We focused on female traits because reproductive investment is more costly and more tightly linked to body size and fitness for females than for males (Wootton, 1985). We examined female TL and wW (spawning + fecundity trials), number and size of spawned embryos (spawning trial), GSI, F_{Pot}, F_{Rel}, maximum oocyte size, as well as stage-specific oocyte sizes (fecundity trial). We also examined the relationships between F_{Pot} and wW using linear regression and compared CO_2 effects on these relationships with analysis of covariance (ANCOVA). Three females were removed from analyses of reproductive trait data because they had either no discernible or only immature oocytes in their gonads. Male trait distributions are presented as supplementary material (Supplementary Figure S1). Statistical analyses were performed in SPSS (V20, IBM).

Results

Spawning trial

Female TL and wW did not differ significantly between duplicates within each CO_2 treatment (control: $Z_{TL} = 0.15$, $Z_{wW} = -0.28$, $p > 0.87$; high CO_2: $Z_{TL} = 1.33$, $Z_{wW} = 1.30$, $p > 0.35$) or between CO_2 treatments when duplicate tanks were pooled ($Z_{TL} = 1.81$, $p = 0.35$; $Z_{wW} = 1.42$, $p = 0.35$, Table 2, Figure 1a and b). Each of the four laboratory populations continually spawned throughout the 60-day period, producing 0.1–3.6 embryos g·female^{-1} during most days, interspersed by several large spawning peaks of 4.8–10.6 embryos g·female^{-1} in three of the four populations (Figure 2a). One high CO_2 replicate did not produce any spawning peaks and thus produced fewer total embryos (174 per female) than the other high CO_2 replicate and both control CO_2 replicates (541–571 per female, Figure 2b, Table 2). The peaks in embryo production showed no apparent periodicity within or synchronicity across tanks. Overall, egg diameter did not differ between duplicates ($Z_{con} = -1.67$, $Z_{high} = 0.93$, $p > 0.35$), nor when pooled, between CO_2 treatments ($Z = -0.47$, $p = 0.82$). Mean (±*SD*) egg diameter across treatments was 1129 ± 52 μm (n = 416).

Fecundity trial

In both CO_2 treatments, female TL and wW increased with temperature ($Z_{TL, con} = 4.96$, $Z_{TL, High} = 4.71$, $Z_{wW, con} = 4.82$,

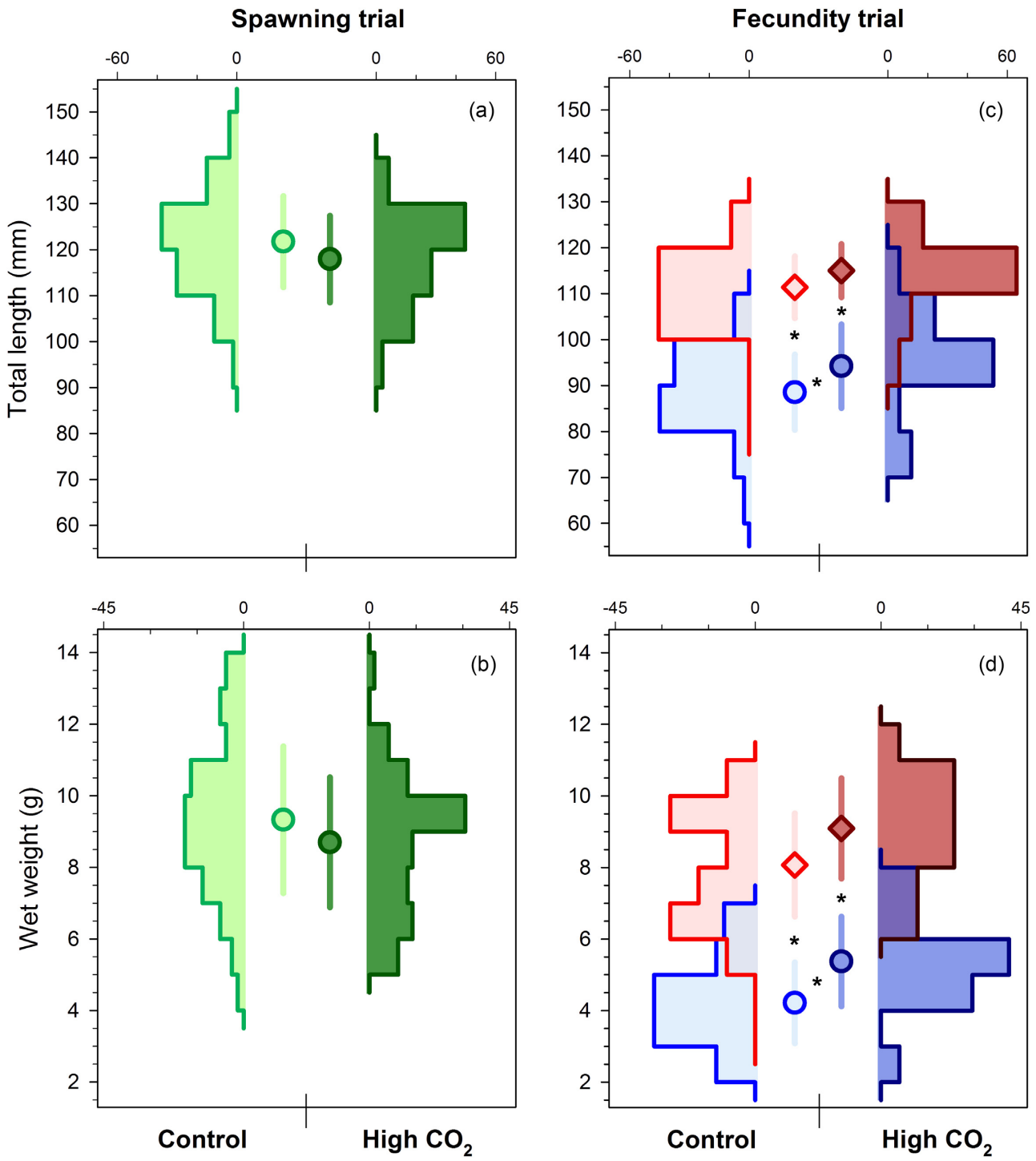

Figure 1. *M. menidia*. Adult female total length (a, c) and wet weight (b, d) distributions after the conclusion of the spawning and fecundity trials at control and high CO_2 conditions at 20°C (a, b—green), 17°C (c, d—blue) and 24°C (c, d—red). Distributions in (a) and (b) were pooled across statistically similar duplicates. Circles and error bars depict treatment-specific means ± 1 *SD*. Asterisks denote statistically significant differences between adjacent treatment groups (Mann–Whitney U tests, $p < 0.05$).

$Z_{wW,\,high} = 4.75$, all $p < 0.001$; Figure 1c–d, Table 2). At 17°C, TL and wW increased with CO_2 ($Z_{TL,\,17°C} = 2.45$, $p = 0.024$; $Z_{wW,\,17°C} = 3.33$, $p = 0.002$), while CO_2 had no effect on body size at 24°C ($Z_{TL,\,24°C} = 1.44$, $p = 0.23$; $Z_{wW,\,24°C} = 1.55$, $p = 0.18$). GSI decreased with temperature at each CO_2 level ($Z_{con} = -3.97$; $Z_{high} = -4.48$, both $p < 0.001$, Table 2, Figure 3a), but CO_2 had no effect on GSI at either temperature ($Z_{17°C} = -1.34$, $p = 0.24$; $Z_{24°C} = 0.31$, $p = 0.82$).

Distributions of F_{Pot} (oocytes/female) and F_{Rel} (oocytes/g female) suggested interacting temperature and CO_2 effects on fe-

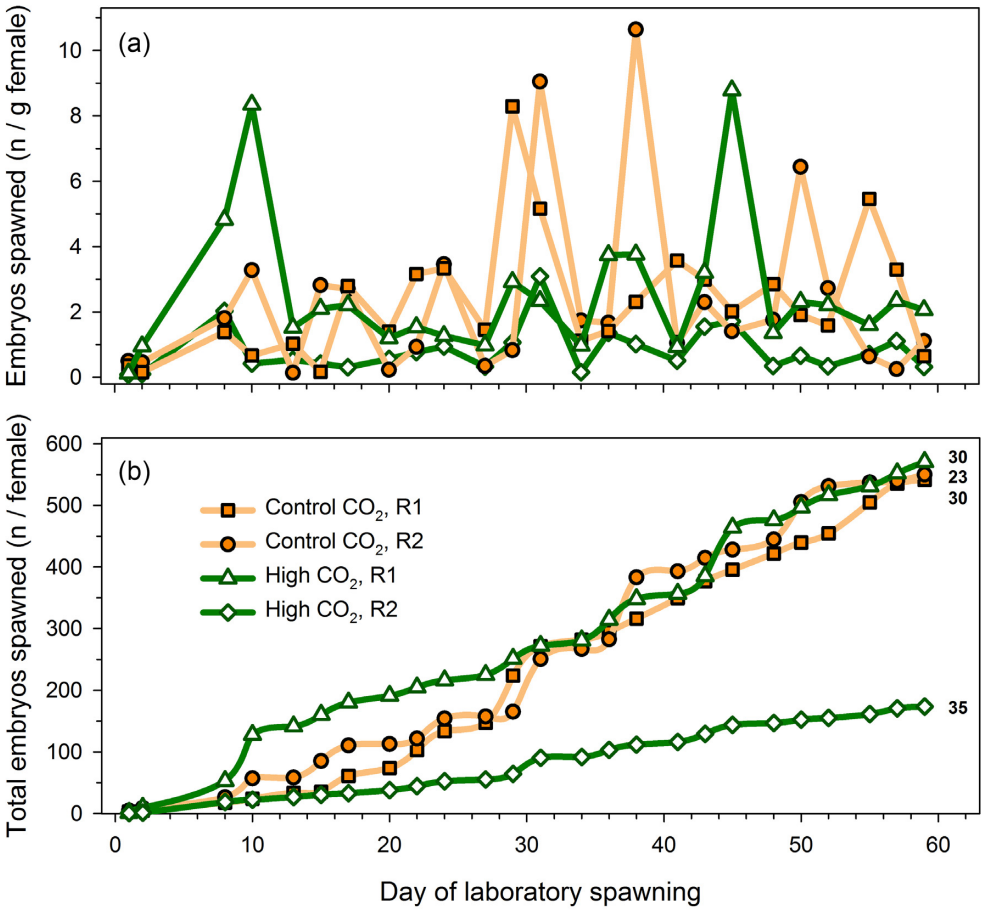

Figure 2. *M. menidia.* Spawning trial: Time-series of embryo production in duplicate laboratory populations (R1, R2) spawning over a 2-month period at control (orange lines, circles and squares) and high CO_2 conditions (green lines, triangles and diamonds), with (a) embryo production per g female and (b) cumulative embryo production per female. Numbers denote females in each duplicate.

male fecundity (Figure 3b and c). At 17°C, high CO_2 increased F_{Pot} ($Z_{17°C} = 2.69$, $p = 0.013$) but had no effect on F_{Rel} ($Z_{17°C} = 0.51$, $p = 0.67$). At 24°C, however, both F_{Pot} and F_{Rel} distributions shifted lower in response to high CO_2 conditions (F_{Pot}: $Z_{24°C} = -2.07$, $p = 0.059$; F_{Rel}: $Z_{24°C} = -3.65$, $p < 0.001$), with average reductions of −19% and −28%, respectively. Warmer temperature negatively affected F_{Pot} at high CO_2 only ($Z_{high} = -4.01$, $p < 0.001$; $Z_{con} = -0.02$, $p = 0.98$; Figure 3b) but reduced F_{Rel} regardless of CO_2 level ($Z_{con} = -4.49$; $Z_{high} = -4.98$, both $p < 0.001$, Figure 3c).

At 17°C F_{Pot} increased linearly with female wW (Linear regression: $F_{Pot} = 871*wW-386$, $r^2 = 0.61$, $p < 0.001$), and this relationship did not differ between CO_2 treatments (ANCOVA: $CO_2 \times wW$, $F_{1,56} = 0.015$, $p = 0.9$, Figure 4). At 24°C, however, F_{pot} increased linearly with wW at only control CO_2 ($F_{Pot} = 413*wW-75$, $r^2 = 0.64$, $p = 0.003$) but not at high CO_2 levels (regression, $p = 0.094$).

Maximum oocyte size distributions were CO_2 independent at each temperature ($Z_{17°C} = -0.67$; $Z_{24°C} = -1.11$, $p > 0.34$), but at high CO_2 treatments they shifted lower at 24°C relative to 17°C ($Z_{high} = -3.13$, $p = 0.002$, Figure 3d). Distributions of temperature- and stage-specific median and maximum oocyte sizes did not differ between CO_2 treatments ($Z_{PG,CA,V1,V2,NM,H} = -1.48$–$1.73$, $p > 0.26$). When pooled, median and maximum oocyte size distributions increased with temperature for some early developmental stages (PG_{max}: $Z = 3.13$, $p = 0.002$; CA_{med}: $Z = 4.04$, $p < 0.001$; $V1_{med}$: $Z = 3.13$; $p = 0.002$) and the final, hydrating stage ($H_{med, max}$: $Z = 2.34$, 2.49; $p = 0.013$, $p = 0.019$). However, the latter is more uncertain, given the low sample size and the observation that many hydrating oocytes in the 17°C treatments collapsed during histological preparation and were therefore not measurable. The same developmental stages (i.e. CA, V1, and H, but not PG, V2, NM) increased in size with wW (all $p < 0.015$); therefore, larger females had larger, more advanced oocyte stages across temperature and CO_2 levels at the end of the experiment (Figure 5).

Discussion

Two complementary rearing experiments examined how long-term and whole life-cycle exposures to future oceanic CO_2 conditions affect female reproductive traits in a temperate marine fish model, the Atlantic silverside. We hypothesized that high CO_2 environments would demand additional metabolic costs due to increased acid-base regulation (Munday *et al.*, 2009; Heuer and Grosell, 2016), thereby diminishing resources for energetically demanding oocyte production, maturation, and spawning (McBride *et al.*, 2015). Our findings partially supported this hypothesis, because potential and relative fecundities shifted lower in response to whole-life, high CO_2 exposure at warm temperatures. At cold temperatures, rela-

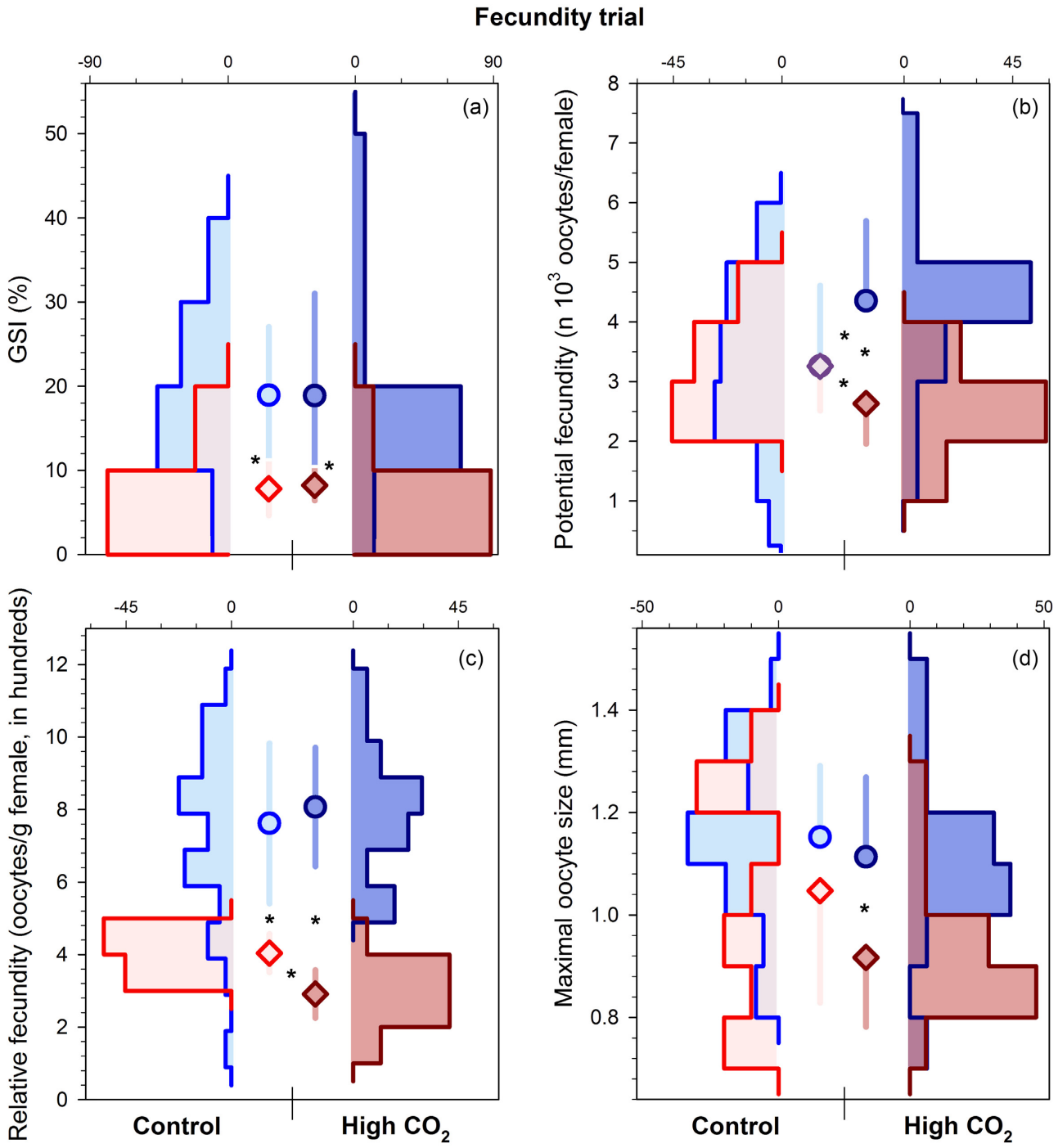

Figure 3. *M. menidia*. Fecundity trial: temperature- and CO_2-specific distributions of (a) gonado-somatic index, (b) potential fecundity, (c) relative fecundity, and (d) maximum oocyte size in spawning-capable females. Blue colors and circles depict 17°C, red colors and diamonds depict 24°C treatments. Symbols and error bars depict treatment-specific means ± 1 *SD*. Asterisks denote statistically significant differences between adjacent treatment groups (Mann–Whitney U tests, $p < 0.05$).

tive fecundity was unaffected, while body size and thus potential fecundity slightly increased with CO_2. Other assessed reproductive traits were sensitive to temperature but not CO_2.

Temperature-dependent CO_2 effects are common in ocean acidification experiments (Munday *et al.*, 2009; Harvey *et al.*, 2013; Przeslawski *et al.*, 2015; Murray and Baumann, 2018), presumably because ectotherms tolerate additional stressors better when near their thermal optimum than when at the extremes of their thermal performance curves (Pörtner, 2008). Yet for silversides, the thermal optimum is often assumed to be around 24°C (Middaugh *et al.*, 1987), whereas 17°C is near their lower thermal limit, which at first seems inconsistent with our results. However, these ther-

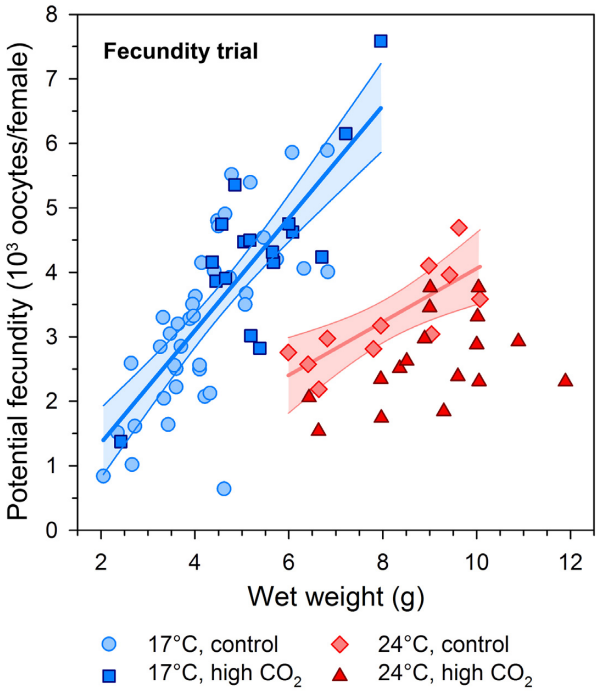

Figure 4. *M. menidia*. Fecundity trial: temperature- and CO_2-specific relationships between female wet weight and potential fecundity. Relationships at 17°C were CO_2-independent; the pooled relationship is plotted ($p < 0.001$). At 24°C, the relationship was significant for the control CO_2 treatment ($p = 0.003$), but not for the high CO_2 treatment ($p = 0.094$). Shading depicts 95% confidence intervals from linear regressions.

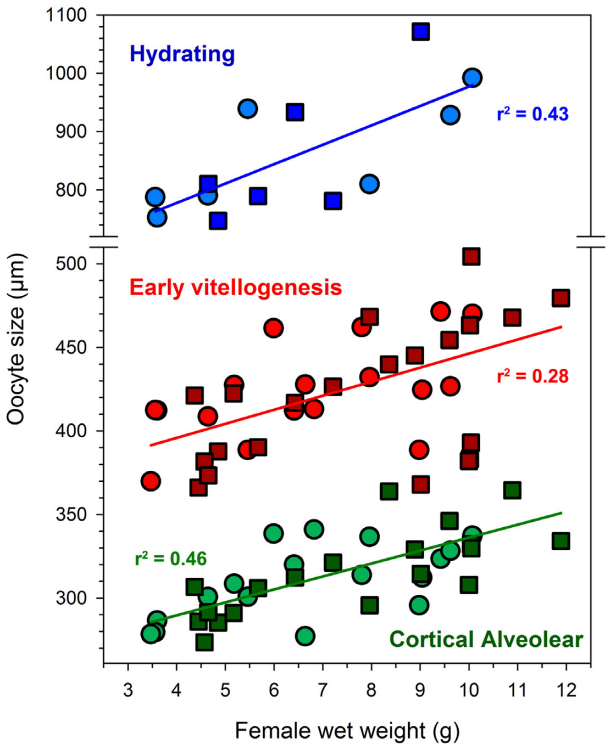

Figure 5. *M. menidia*. Fecundity trial: relationships between wet weight and stage-specific median oocyte size in spawning capable females. Each symbol represents an individual female, with circles and squares depicting females reared at control and high CO_2 levels, respectively.

mal optima pertain to somatic growth, not reproductive traits, and they have largely been derived from rearing larvae and juveniles (Conover and Present, 1990), which are adapted to developing in warm nearshore waters during summer and fall (Conover and Ross, 1982). Indeed, our growth data confirmed that silversides grew 24% larger and 79% heavier at 24°C than at 17°C, consistent with expectations based on thermal growth reaction norms. But sub-adults in the wild mature at much colder temperatures while overwintering offshore (Conover and Murawski, 1982), after which they return to nearshore waters and commence spawning as temperatures reach approximately 15°C again (at ~41°N, Pringle and Baumann, 2019). This means that our cold temperature treatment of 17°C was actually closer to the natural thermal conditions for maturing silversides than the warm 24°C treatment, which may explain why the latter not only led to lower reproductive investment (lower GSI) but also to significantly lower potential and relative fecundities in the presence of the additional stressor, high CO_2.

Conversely, high CO_2 increased potential fecundity at 17°C, but only because body size increased with CO_2, and larger females simply produced more oocytes. Oocyte production per unit weight remained unchanged at 17°C, as indicated by the linear relationship between weight and potential fecundity, and confirmed by CO_2-independent relative fecundities. At 24°C, however, the relationship between weight and potential fecundity disappeared at high CO_2 conditions, body size was independent of CO_2 treatment, and both potential and relative fecundities declined at high compared to control CO_2 conditions—suggesting a size-independent CO_2 effect. We note that the spawning trial at an intermediate temperature (20°C) found neutral CO_2 effects on embryo production, which is consistent but circumstantial evidence, because the two trials are not directly comparable. We infer that high temperature combined with high CO_2 conditions led to increased metabolic demands that compromised available resources for oocyte maturation in laboratory silverside populations.

Our findings contrast with the still limited empirical evidence that has so far suggested mostly stimulating effects of increased CO_2 on fish reproductive output. Pioneering work quantified breeding in wild-caught cinnamon clownfish (*Amphiprion melanopus*) over a 9-month period, reporting twice as many pairs breeding, producing twice as many clutches per pair and 67% more eggs per clutch in elevated compared to control CO_2 treatments (~400 vs. ~1000 µatm, Miller *et al.*, 2013). A follow-up study confirmed a CO_2-mediated increase in egg clutches and eggs per clutch for the congener *Amphiprion percula*, although it also found contrasting, negative CO_2 effects on produced egg clutches in a different reef fish, *Acanthochromis polyacanthus* (Welch and Munday, 2016). Schade *et al.* (2014) acclimated groups of adult three-spined sticklebacks (*Gasterosteus aculeatus*) caught in the German Bight (North Sea) to control and high CO_2 conditions (~1000 µatm) for three months, after which strip-spawning revealed a 20% increase in eggs per female from control to high CO_2 levels, despite similar female body size. In two-spotted gobies (*Gobiusculus flavescens*), wild-caught breeding pairs held for 4 months under high CO_2 levels (~2300 µatm) produced 50% more clutches and 44% more eggs per clutch than pairs under control conditions, although there was a trade-

off with smaller hatch size (Faria *et al.*, 2018). In all these experiments, breeding pairs were provided high or *ad libitum* food rations, which might have allowed females to increase consumption to sustain their CO_2-stimulated reproductive output. This point was recently underscored by Nagelkerken *et al.* (2021), who measured prey availability and reproductive investment in several small benthic fishes (triple-fin gobies, blenny) inhabiting contrasting CO_2 environments at natural CO_2 vents off the coast of New Zealand. Over several years, the authors found increased benthic prey abundance at vent sites compared to control sites, which coincided with higher foraging activities and higher reproductive investment in both male and female fishes (Nagelkerken *et al.*, 2021).

Interestingly, whole-life silverside rearing resulted in neutral (24°C) to weakly positive CO_2 effects (17°C) on adult female body size. This seems inconsistent with the neutral to negative growth responses to high CO_2 documented by two previous studies, which reared larger numbers of silversides to late juvenile stages (to ∼130 dph) at comparable CO_2 and temperature levels (Murray *et al.*, 2017; Murray and Baumann, 2020). Contrasting intraspecific and across-experiment outcomes are not uncommon in ocean acidification studies (Parker *et al.*, 2011), and serial experimentation is often required for robust quantification of trait responses (Baumann *et al.*, 2018). In silverside experiments, additional variability is likely introduced by using wild-caught parental founders that may vary in genetic composition within and between spawning seasons (Murray *et al.*, 2014; Malvezzi *et al.*, 2015). It is also possible that positive CO_2 effects only arise after the period of fast larval and juvenile somatic growth ends, and fish start diverting resources towards reproduction and thus grow slower. For silversides in the wild, this coincides with seasonal declines in temperature and food availability (Schultz *et al.*, 1998), whereas laboratory populations experienced static thermal conditions and unchanged ∼20% food rations. These food rations might be limiting larval and juvenile growth and therefore produce CO_2-induced growth reductions, but in maturing sub-adults they might have provided sufficient excess resources to benefit preferentially the fish maturing at high CO_2 treatments (Supplementary Figure S2).

Over the past decade, a large number of studies systematically examined many Atlantic silverside traits for their climate sensitivity, using short- and long-term as well as single- and multi-stressor exposures to static and fluctuating regimes of CO_2, temperature, and oxygen (e.g. Murray *et al.*, 2014; Depasquale *et al.*, 2015; Malvezzi *et al.*, 2015; Miller *et al.*, 2016; Baumann *et al.*, 2018; Cross *et al.*, 2019; Murray and Baumann, 2020; Schwemmer *et al.*, 2020). Silversides are appealing models, given their ecological importance as forage fish, their almost year-round, nearshore availability to experimenters, their short life span, and the existence of established laboratory spawning and rearing protocols (Middaugh *et al.*, 1987). They thus were also obvious candidates here for studying reproductive CO_2 sensitivities. However, the species also exhibits life history characteristics that are difficult to overcome experimentally and which limit conclusions particularly with regards to the effects of environmental conditions on reproduction.

As an obligatory schooling species, silversides must be held in large groups (demanding large enclosures, replication trade-offs) for quantifiable, semi-natural spawning activity to occur. This can be insightful (spawning trial) but it precludes resolving individual female contributions. We first tried to overcome this by experimenting with small silverside groups (female + three males), but this failed to elicit quantifiable spawning. The alternative was to quantify maturing oocytes in individual female gonads prior to spawning (fecundity trial), which provided a useful measure of potential fecundity, but not necessarily of realized fecundity that is ultimately of most interest. For example, realized embryo production during the spawning trial (∼600 embryos female^{-1}) was five to seven times lower than potential fecundities estimated during the fecundity trial, which may be due to method differences between trials, but could also indicate that a large number of vitellogenic oocytes are resorbed again after spawning. In total spawners, i.e. fish species that release all eggs in one spawning event, potential and realized fecundity are tightly correlated (McBride *et al.*, 2015), but silversides are batch spawners that mature and release egg batches in semi-lunar intervals over the course of their spawning season in the wild (Conover, 1985). Moreover, overall oocyte size-distributions (Supplementary Figure S3) and stage-specific oocyte measurements showed overlapping size ranges of successive developmental stages, therefore suggesting that silversides are indeterminate batch spawners with asynchronous oocyte development (Ganias *et al.*, 2015; Ganias and Lowerre-Barbieri, 2018), where the number of maturing batches cannot be readily deduced from distinguishable oocyte cohorts. Asynchronicity might partially be an artifact of laboratory populations lacking periodic environmental cues (temperature, tides, moon light), which is supported by the aperiodic and asynchronous spawning patterns observed during the spawning trial (Conover and Kynard, 1984). A final difficulty in conducting whole-life experiments with silverside populations from mid-latitudes is their high degree of temperature-dependent sex determination (Conover and Kynard, 1981; Murray and Baumann, 2020), which produces roughly balanced sex ratios at low temperatures (i.e. 17°C), but results in impractically small female proportions at warmer temperatures (at 24°C, only 1–2 out of 10 fish become females).

In conclusion, our two complementary approaches suggested neutral CO_2 effects on silverside reproductive traits at low temperatures, but CO_2-induced reproductive depression at warm temperatures, which currently occur only during summer and not during overwinter maturation. Many reproductive traits depended on body size, thereby emphasizing the fitness relevance of growth and the general importance of examining growth-determining variables in climate sensitivity studies of fish. The short, annual life cycle of this species may also allow evolutionary responses to future high CO_2 environments (Malvezzi *et al.*, 2015), thereby potentially offsetting any temperature × CO_2-releated reductions in fecundity. Future work should aim for further in-depth reproductive analyses, enhanced replication and statistical power, as well as seasonally varying thermal regimes. However, conclusive insights will likely depend on developing an alternative, non-schooling fish model that is a total broadcast spawner equally amenable to experimental manipulation.

Ethics

The Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut approved fish husbandry and experimental protocols (#A14-032, #A17-043).

Supplementary Data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Data availability

Citable source data are available from the BCO-DMO database: Spawning trial DOI: 10.26008/1912/bco-dmo.845633.1, 10.26008/1912/bco-dmo.845804.1; Fecundity trial DOI: 10.26008/1912/bco-dmo.845906.1, 10.26008/1912/bco-dmo.845921.1

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