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Original Article

No effect of elevated carbon dioxide on reproductive behaviors in the three-spined stickleback

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Received 3 February 2017; revised 8 July 2017; editorial decision 18 July 2017; accepted 2 August 2017.

Ocean acidification, the reduction in ocean pH resulting from anthropogenic emissions of carbon dioxide (CO₂), has been predicted to alter the behavior of fishes. During experimental exposure to CO₂ concentrations projected for the year 2100 (~1000 μatm), fish have been reported to display disturbances in activity, learning, behavioral lateralization, and even attraction to predator cues. Reproductive behaviors have received far less attention, despite an intensive research effort on ocean acidification and its ecological importance. Here, we investigate whether elevated levels of CO₂ affect reproduction in breeding pairs of the three-spined stickleback, *Gasterosteus aculeatus*, a model species in behavioral, evolutionary ecology, and environmental toxicology. We found that males under both present day levels (400 μatm) and future levels (1000 μatm) of CO₂ developed normal sexual ornaments, pursued normal nest building activities, exhibited similar levels of courtship behaviors and displacement fanning, and had the same mating probability. Moreover, fanning behavior during the paternal care period followed what is expected for the species for males from both treatments, and there was no effect of treatment on the numbers of offspring produced. This study is the first to investigate the effect of elevated CO₂ on the complete breeding cycle in detail, studying an array of highly fitness-relevant traits. Our study showing surprising resilience of fish reproduction is an important contribution in order to realistically predict the impacts of future ocean acidification.

Key words: Climate change, courtship, *Gasterosteus aculeatus*, ocean acidification, sexual ornamentation, teleost.

INTRODUCTION

The increasing concentration of atmospheric carbon dioxide (CO₂), the primary greenhouse gas emitted through human activities, decreases ocean pH in a process commonly referred to as ocean acidification (Caldeira and Wickett 2005). When CO₂ enters the ocean, it increases the partial pressure of CO₂ (pCO₂) causing hypercapnia. The CO₂ reacts with water forming carbonic acid (H₂CO₃) that disassociates into bicarbonate (HCO₃⁻) and (H⁺), which reduces pH. The component of the altered carbonate chemistry causing the biological effects may depend on the type of organisms, but the pCO₂ is thought to be the major driver in fish (Melzner et al. 2009). CO₂ manipulation experiments have

reported effects on behavior and physiology of marine organisms (Ishimatsu et al. 2008; Dupont et al. 2010; Heuer and Grosell 2014). In a review by Ishimatsu et al. (2008), it was pointed out that the possible implications of elevated CO₂ on reproduction needed urgent attention. With more than 300 publications per year, the research effort devoted to ocean acidification (OA) is unprecedented in marine sciences (Browman 2016). Despite this, the lack of studies on the potential effects of OA on reproduction, fish reproduction in particular, has been emphasized by the scientific community (Heuer and Grosell 2014).

To date, there are no studies investigating the effects of elevated CO₂ on all aspects of the complete breeding cycle of fish. The few studies that exist have typically investigated the effects of CO₂ on reproductive output, and they offer little consensus towards general conclusions. For example, elevated CO₂ has been suggested to stimulate reproductive output, as adult cinnamon

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anemone fish, *Amphiprion melanopus*, kept at elevated CO₂ produced more clutches per breeding pair and more eggs per clutch (Miller et al. 2013). An increase in egg production was also reported for breeding pairs of the three-spined stickleback, *Gasterosteus aculeatus*, held at elevated levels of CO₂ (Schade et al. 2014). However, a later study (also investigating the effect of warming), again using the cinnamon anemone fish (*A. melanopus*), reported essentially no effects of CO₂ on number of clutches produced per pair or number of eggs per clutch (Miller et al. 2015). Similarly, no difference in number of eggs laid in ambient or high CO₂ nests were reported for ocellated wrasse, *Symphodus ocellatus*, habituating a natural CO₂-seeps site nearby Vulcano Island in Italy (Milazzo et al. 2016). A lack of CO₂ effect on clutch size has also been reported in the temperate two-spotted goby, *Gobiusculus flavescens*, however notably after keeping breeding pairs for only 24–48 h in elevated CO₂ (Forsgren et al. 2013). A study using the broad-nosed pipefish, *Syngnathus typhle*, similarly found no effect of acute exposure (4.5 h) to low pH on male pouch fullness (i.e. equivalent to clutch size) (Sundin et al. 2013). Except for the apparent need to further examine the effect of elevated CO₂ on reproductive output due to the lack of consensus amongst published results, the possible implications of elevated CO₂ on additional elements of reproduction, such as sexual ornaments, mate choice, courtship, and parental care, remain virtually unexplored. A study by Milazzo et al. (2016), however, investigated the effects of elevated CO₂ on courtship intensity, nest defense from sneaker males, number of males and females present at the nest, and male-male competition, in the ocellated wrasse inhabiting natural CO₂-seeps, and report no effect of elevated CO₂. They however report that the number of spawning events involving only one female and one male was lower under high CO₂ (Milazzo et al. 2016). Taken together, these results suggest that reproductive output and reproductive behaviors might be relatively robust to high CO₂, although general conclusions cannot be made until further studies have been conducted.

Here we investigated the impact of elevated CO₂ on male sexual ornaments, precopulatory behaviors, and paternal care. We used the three-spined stickleback, *G. aculeatus*, which has been a model organism in behavioral and evolutionary biology for more than 4 decades. Since the genome of the three-spined stickleback is sequenced, it has been proposed that this species could serve as a temperate model species for ocean acidification research (Lai et al. 2015). Despite the fact that this species inhabits a variety of environments, showing strong physiological plasticity and potential for acclimation to stressors (Pottinger et al. 2002; Östlund-Nilsson 2007; Barrett et al. 2011), some studies report that the three-spined stickleback can be affected by elevated CO₂, with potential implications such as increased escape time (Jutfelt et al. 2013), decreased curiosity (Jutfelt et al. 2013), and altered behavioral lateralization (Jutfelt et al. 2013; Lai et al. 2015; Näslund et al. 2015). The reproductive biology of this species is well described (reviewed in (Östlund-Nilsson 2007), and suitable for behavioral experimentation as the majority of behaviors can be quantified by a human observer. At the beginning of the breeding season, males leave the shoal to settle in shallow areas, establish a territory and build a nest. During this time males develop sexual ornaments consisting of an orange jaw and shiny blue eyes (Supplementary Figure S1), with the former being important for attracting females (Kraak et al. 1999; Flamarique et al. 2013), and functioning as a honest fitness signal (Barber et al. 2001a; Candolin and Tukiainen 2015). When nest construction is completed, the male begins to attract females through a characteristic zigzag courtship dance and attempts to

lead the female to the nest by swimming rapidly to the nest. At the nest, the male engages in nest activities, primarily fanning behavior, so called displacement fanning (Östlund-Nilsson 2007). If the female does not follow the male to the nest, the male returns to her to perform more zigzag bouts and leads back to the nest. When the female has laid her eggs in the nest she leaves, or is chased off by the male, who then cares for the eggs until they hatch. Male care consists of nest defense, nest-maintenance behaviors, removal of dead and diseased eggs, and oxygenation of embryos by fanning movements of the pectoral fins. In this study, we focused on investigating male sexual ornaments, nest-building behaviors, courtship, reproductive output, and paternal care, since these are some of the major components of stickleback reproduction.

Given that previous studies report that elevated CO₂ may affect behaviors in stickleback (Jutfelt et al. 2013; Lai et al. 2015; Näslund et al. 2015), we hypothesized that also reproduction would be affected. However, since alterations with likely positive functional effects, in the form of increased egg production and increased juvenile survival and growth rates, have been reported (Schade et al. 2014), the directions of change of different reproductive traits are difficult to predict. In this experiment, we compared reproductive performance of control fish (exposed to pCO₂ ~400 µatm) to fish exposed to elevated pCO₂ (1000 µatm). We measured male sexual ornamentation, male nest-building behaviors, male and female courtship behaviors, reproductive output, and paternal care in 56 individually housed reproductive couples.

METHODS

The experiments were performed at Sven Lovén Centre for Marine Infrastructure, University of Gothenburg, Kristineberg, on the west coast of Sweden during May–June in 2014. Adult three-spined stickleback were collected using seine-net in bays of the Gullmar fjord, nearby the research center (58°15'N, 11°28'E). The fish were caught in May 3–8, 2014, before the onset of the breeding season. In the laboratory, the fish were initially housed in groups in 4 flow-through holding aquaria (62 × 39 × 36 cm [length, width, height], water depth: 30 cm), sexes separated (keeping the sexes separated is common practice and ensures that no animal had mated recently prior to the experiments, Östlund-Nilsson 2007). Artificial plants were provided for shelter. Fish were fed frozen aquarium food (*Artemia* spp. nauplii, mosquito larvae and Mysids) twice per day. Temperature and salinity followed natural conditions in the area (temperature: 10.9 ± 0.62 °C, salinity: 23.6 ± 0.90 [mean ± SD]). The light cycle was set to 16:8 h light:dark to mimic natural conditions. The research detailed in this paper was performed according to current national legislation on Animal Welfare and the experimental procedures were approved by the Animal Ethics Committee in Gothenburg (Dnr 151–2011 and 103–2014).

CO₂ exposure

Methods of CO₂ exposure were similar to (Sundin and Jutfelt 2016, for details, see Electronic Supplementary Material). Briefly, the exposure aquaria (38 × 36 × 35 cm, males divided over 4 aquaria, 2 per treatment and females over 8 aquaria, 4 per treatment) had a constant supply of flow-through control or elevated CO₂ seawater (setup of header tanks and aquaria followed a randomized block design according to (Hurlbert 1984; Cornwall and Hurd 2015). The target value of 1000 µatm for the CO₂-treatment was maintained using pH stat computers (Aqua Medic, Bissendorf, Germany) connected to solenoid valves regulating the administration of 100%

CO₂ gas (AGA, Sweden) into the header tanks. The pCO₂ of the exposure tanks was measured daily using a handheld CO₂ meter (GM70, Vaisala, Finland) connected to a submerged gas-permeable PFTE probe (Qubit systems, Kingston, Canada) as described previously (Jutfelt and Hedgärde 2013; Sundin and Jutfelt 2016) (i.e. direct pCO₂ measurements). Mean pCO₂ for the CO₂-treatment was ~1000 μ atm, and ~400 μ atm for the control treatment (Supplementary Table S1). Temperature was measured daily (~10–13 °C for both treatments, Supplementary Table S1). Data on salinity levels were derived from the continuous monitoring of the flow-through system at the Kristineberg marine Station, and was ~25 PSU for both treatments (Supplementary Table S1). The same feeding regime and light cycle as during the holding period was applied during the exposure. Artificial plants as well as brown algae (*Fucus vesiculosus* and *F. serratus*) were provided to all fish for shelter in the exposure tanks. Absence of suitable nesting material in the male aquaria discouraged nest-building behavior amongst males. All fish were placed in the exposure tanks at the same time and used in the reproduction experiment in 2 runs (due to space limitations of reproduction aquaria), thus resulting in a minimum of 14 days, and maximum of 34 days CO₂ exposure in total prior to the commencement of behavioral observations (this was accounted for in the analyses, see statistical analysis section below).

Experimental design

To determine the effect of increased levels of CO₂ on reproduction, males were transferred to individual flow through mating aquaria (30 × 30 × 25 cm, water depth 25 cm), equipped with one artificial plant for shelter (following Candolin 1997), while continuing the CO₂ exposure using the same method as described above (randomized block design: (Hurlbert 1984; Cornwall and Hurd 2015), Supplementary Table S1). Prior to transferring the males from exposure to individual tanks, they were photographed (see photography of male ornaments and image analysis section below). Length (mm) and weight (g) was measured immediately after the photography and there was no difference between treatments ($n = 28$ males per treatment, mean \pm SD, length: CO₂ 55.9 \pm 5.4,

control 54.4 \pm 4.8, $t_{54} = 1.10$, $P = 0.277$; weight: CO₂ 1.58 \pm 0.5, control 1.36 \pm 0.5, $t_{54} = 1.71$, $P = 0.093$). After 3 days of acclimation to the individual aquaria (i.e. minimum 14 days (run 1), maximum 34 days (run 2) of CO₂ exposure in total prior to behavioral observations commenced), all males were provided with a nesting dish (\varnothing 14 cm), filled with 1 cm of sand and filamentous green algae (*Cladophora* sp.) for nest construction (following Candolin 1997). Male nest building intensity (building or not building) was visually observed in real time for 15 min at 5 occasions (Figure 2). To stimulate nest building, and to investigate male courtship behaviors during the nest-building phase, each male was provided with a stimuli female (Frischknecht 1993) (Figure 1). During 15 min (for 3 consecutive days), male courtship behaviors were observed, noting zig-zag, displacement fanning, gluing on nest and crawling through nest, thus providing a value of courtship intensity (0–4) based on number of courtship behaviors performed by the male.

When nest building was completed, males were provided with a mating female. Females were weighed and then introduced to the males enclosed in a non-sealed see-through Plexiglas cylinder for 5 min and thereafter released into the male tank and allowed to interact and spawn with the male. To ensure usage of females that were ready to mate we noted whether females performed the head-up posture, an indicator of readiness to mate (Mayer and Páll 2007). Most females performed the head-up posture and there was no difference between treatments (during time in cylinder: 11 CO₂, 14 control, out of 18 females respectively; $\chi^2_{1, 34} = 1.19$, $P = 0.275$, when released: 15 CO₂, 16 control, out of 18 females respectively; $\chi^2_{1, 34} = 0.23$, $P = 0.629$). During the 5 min, the female was enclosed in the cylinder, male behaviors were quantified (courtship zig-zag duration, displacement fanning duration, number of leads to the nest, number of times gluing on the nest) according to (Östlund-Nilsson 2007). After the initial 5-min observation, the cylinder enclosing the female was carefully removed and the male and female were allowed to freely interact and mate for 25 min. We quantified the same behaviors as when the female was enclosed in the cylinder, and in addition measured latency until first mating attempt, number of mating attempts, latency until successful mating

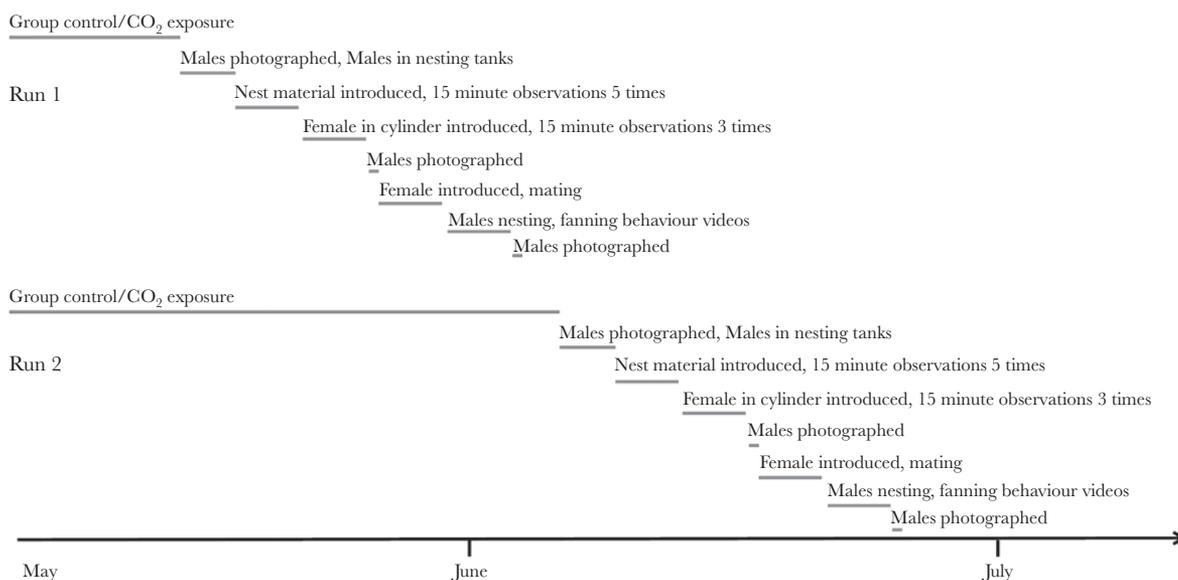


Figure 1

Timeline of the experimental design for runs 1 and 2, showing group control/CO₂ exposure, male photography events, nest building phase, mating, and parental care phase.

event, mating duration, and counted number of bites at the female (Östlund-Nilsson 2007). If spawning occurred, the female was immediately removed, weighed again and in addition we measured length (there was no difference in female length or weight between treatments, mean \pm SD, length: CO₂ 60.9 \pm 7.1, control 60.1 \pm 6.3, $t_{34} = 0.40$, $P = 0.693$; weight: CO₂ 2.1 \pm 0.8, control 2.0 \pm 0.8, $t_{34} = 0.10$, $P = 0.922$). If no mating occurred during the maximum observation period, the female and male were left undisturbed for 1 h. If no mating had occurred during this time (as determined by carefully checking the nest for eggs) the female was removed and the male was provided with a new female one day later. Males were given a maximum of 4 different females, if no spawning had occurred after the fourth female the male was excluded from further experiments (there was no difference between treatments in number of females required for mating, mean \pm SD: CO₂ 2.1 \pm 1.1, control: 2.3 \pm 1.0, $\chi^2_{1,34} = 0.42$, $P = 0.517$). Eighteen males per treatment completed spawning (further details on excluded males, see Supplementary Material).

To investigate possible effects of elevated CO₂ on paternal care we recorded nest-fanning behavior. Each male was filmed in HD (using a Nikon J3 and a Panasonic HC-V130) once per day for 15 min, starting 2 days after the male had mated and continuing until 8 days after receiving eggs (Figure 1). Videos were subsequently analyzed by manually measuring total time (s) spent fanning during the 15-min recording. Nests were checked visually to determine when the eggs had hatched. Two days after hatching, the male was removed from the tank and all offspring were collected and counted. No offspring was found for 8 of the males that had mated (4 males per treatment), leaving a total of 14 males per treatment for the fanning analysis.

The total setup was replicated twice (run 1: May 20–June 8; run 2: June 9–28, Figure 1), using 14 males per treatment per run. Males were fed frozen *Artemia* spp. nauplii once per day throughout the courtship experiment. The aquaria were not cleaned to minimize disturbance. Temperature was controlled and, for the first run, initially set to 16 °C (i.e. close to the natural temperature at that time) and slowly increased to 18 °C to avoid a decrease in reproductive behaviors due to a possible drop in temperature (over a period of 13 days). For the second run, temperature was maintained at 18 °C (the natural temperature during run 2). pCO₂ was carefully measured in the male aquaria once per day using the Vaisala pCO₂ meter as described above (Supplementary Table S1).

Photography of male ornaments and image analysis

To investigate the possible effect of elevated CO₂ on male sexual ornaments, males were photographed using a Canon 5D mkII DSLR camera and 2 Viewpoint LED white light ramps (Viewpoint, Lyon France) for even and constant illumination, with a background including a color and grey scale palette standard for later color measurements (Supplementary Figure S2). All males were photographed 3 times (Figure 1). The digital images were analyzed using Adobe Photoshop 6.0 (Adobe Systems Inc., Mountain View, CA, USA), in accordance to (Svensson et al. 2005). In brief, the images were first converted to CIE Lab colors with the scales 0 to 255 with neutral color at 128 and then *L* (lightness) was normalized by setting the black circle in the image to zero and the white reference box to 255 (Supplementary Figure S2). The mean values of *a* (green to red) and *b* (blue to yellow) of the selected areas (dorsal half of iris and jaw) (Supplementary Figure S2) were measured using the histogram tool. With these principles, an orange jaw has

high *a* and *b* values whereas a blue iris has a low *b* value and neutral or low *a* value. The variation in the reference images within each photo event was low (Supplementary Table S2). Image analysis was conducted blindly regarding treatment groups (by H.N.S.).

Statistical analyses

To determine if elevated CO₂ had an effect on male sexual ornaments, we analyzed the values from the image analysis from each photo event separately, since the occasions were separated by several days, and since the first photo was taken under slightly different conditions as compared to photo event 2 and 3, using GLMs (Poisson distribution, log link function). Iris *a* and *b*, and jaw *a* and *b*, were the response variables respectively, and treatment (control, CO₂), run (1, 2), and their interaction as fixed effects. Male weight was included as a covariate.

To test whether the CO₂ treatment had an effect on nest-building activity (observed at 5 occasions, building denoted a value of 1, not building 0) and male courtship intensity (scoring courtship intensity 0–4 based on number of courtship activities observed on 3 occasions) during the nest-building phase we used generalized linear mixed models (GLMMs) with Laplace approximation and binomial error structure (Zuur et al. 2009). Treatment (Control and CO₂), run (1 and 2), observation event (nest building: 1–5, courtship: 1–3) and their interactions were entered as fixed factors, while replicate (male ID) was included as a random factor.

To test whether CO₂ treatment had an effect on courtship behaviors directed towards the mating female, we used generalized linear models (GLMs, Poisson distribution, log link function). We analyzed courtship behaviors directed towards the female when enclosed in the cylinder as well as when the female was free. Zig-zag duration, displacement fanning duration, number of leads to the nest, and number of times gluing at nest, respectively, were the response variables for the analyses of female enclosed in cylinder. When the female was free we analyzed all of the above, and in addition the number of bites at female, latency until first mating attempt, number of mating attempts, latency until mating, mating duration, and number of offspring subsequently collected. For the analyses of female in cylinder we included treatment (CO₂ and control) and run (1 and 2) as well as their interaction as fixed effects and for female free we included male and female weight in addition as covariates.

To investigate whether elevated CO₂ had an effect on paternal care, we fitted a linear mixed effects model (LMM) to the mean fanning duration (s min⁻¹). We included treatment (control and CO₂), run (1 and 2), and incubation day (day 2 to 8) as fixed factors, number of offspring and their interactions as fixed factors, and replicate (male ID) as a random factor.

Due to the large number of tests on the same dataset for some of the analyses (14 tests for the analysis of courtship and mating, and 12 tests for the analysis of male sexual ornaments) we used the Benjamini and Hochberg False Discovery Rate (FDR) correction to adjust significant full model *P* values (Benjamini and Hochberg 1995) (Supplementary Tables S3 and S4). For the minimal-adequate model, non-significant interactions (α -level: 0.10, or α -level: 0.05, when AIC showed a better model fit when excluding interactions > 0.05) and main effects (α -level: 0.05) were dropped using stepwise backward exclusion. Dropped interactions and main effect were again included in the final model, one at a time, to verify that they did not have a significant effect. Minimal adequate models are presented in the manuscript (Tables 1–4), while full models can be found in the Supplementary Tables S5–S8. JMP 11 (SAS Institute

Inc., Cary, NC, USA) was used for GLMs and *t*-tests, while R version 3.2.3 (R Core Team 2013) and R package “lme4” (Bates et al. 2015) was used for the GLMMs and LMMs. For the GLMMs, *P* values were given by default (Bates et al. 2015), and for the LMMs the package lmerTest was used to derive *P* values (Kuznetsova et al. 2016).

RESULTS

Precopulatory behaviors

The analysis of nest-building frequency revealed that there was a significant interaction between observation event and run (Table 1), with males from run one having a lower nest building frequency during the first observation events compared to males from run 2, while this relationship was the opposite during the later observation events (Supplementary Figure S3). There was, however, no effect of treatment, and additional 2- and 3-way interactions were non-significant (full model presented in Supplementary Table S5). The analysis of courtship intensity during the nest-building phase revealed an increase in courtship intensity over time (Table 1). There was however no effect of treatment (Supplementary Table S5).

Courtship and mating

The analysis of courtship and mating showed that larger males had a higher zig-zag duration when the female was released

Table 1

The effect of treatment (high CO₂ and control), run (1 and 2), observation event (5 for nest building and 3 for courtship), and the interactions, on nest building frequency and courtship intensity during the nest-building phase

Response variable	Explanatory variable	Estimate (± SE)	<i>z</i>	<i>P</i>
Nest building	Intercept	-0.03 (0.59)	-0.05	0.960
	Run	2.24 (0.87)	2.56	0.011
	Obs. event	0.31 (0.16)	1.95	0.051
	Run*Obs. event	-0.87 (0.23)	-3.75	< 0.001
Courtship	Intercept	-2.79 (0.56)	-4.96	< 0.001
	Treatment	-0.83 (0.53)	1.55	0.122
	Run	0.99 (0.54)	1.85	0.064
	Obs. event	0.38 (0.14)	2.77	0.006

Parameter estimates are from GLMMs with replicate (male ID) as a random factor. *n* = 28 males per treatment. All estimates are given as contrasts to the intercept, and only the minimum adequate model is presented (for full model, see Supplementary Table S5).

Table 2

The effect of treatment (high CO₂ and control), run (1 and 2), and their interaction, and male and female weight on the courtship behavior zig-zag during the time when the female was released into the male tank (female free), and on number of offspring (*n* = 18 males per treatment)

Response variable	Explanatory variable	Estimate (± SE)	DF	χ^2	<i>P</i>
Zig-zag (s) female free	Intercept	1.82 (0.93)		3.41	0.065
	Treatment	0.22 (0.39)	1, 31	0.33	0.563
	Run	0.68 (0.54)	1, 31	1.47	0.225
	Treatment*Run	-1.11 (0.53)	1, 31	5.16	0.023
	Male weight	1.25 (0.48)	1, 31	6.41	0.011
# offspring	Intercept	2.62 (0.45)		32.03	< 0.001
	Treatment	-0.19 (0.13)	1, 32	2.36	0.125
	Male weight	0.55 (0.23)	1, 32	5.30	0.021
	Female weight	0.44 (0.13)	1, 32	11.56	0.001

Parameter estimates are from GLMs and are given as contrasts to the intercept. Only the minimum adequate models are presented (for full models, see Supplementary Table S6).

from the cylinder (Table 2). For this analysis there was also a significant interaction between run and treatment (Table 2), showing that the control fish had a higher zig-zag duration in the second run, whereas the CO₂ fish had a higher zig-zag duration in the first run (mean ± SE, Control run 1: 28.3 ± 8.7, Control run 2: 132.5 ± 97.3, CO₂ run 1: 89.6 ± 36.5, CO₂ run 2: 40.3 ± 17.1). Larger males and females also produced a greater number of offspring (Table 2). Although we did not measure number of eggs laid in the nest, the positive correlation between number of offspring collected and weight reduction (difference in weight before being placed in the mating tank and after mating) in females lends support to the assumption that number of offspring collected is an accurate measure of the actual number of eggs initially laid (*r* = 0.50, *t*₃₄ = 3.37, *P* = 0.002). For some of the males that mated, no offspring was found (4 CO₂ and 4 control males) and 1 control male was observed to eat several of the eggs in his nest just after mating, thus resulting in only 16 larvae subsequently collected. Removing these 9 replicates gave a similar result (Supplementary Figure S4).

The CO₂ treatment had no significant effect for any of the response variables investigated (i.e. zig-zag, displacement fanning, leads to nest, times gluing at nest, number of bites at female, latency until first mating attempt, number of mating attempts, latency until mating, mating duration and number of offspring), regardless whether the female was enclosed in the cylinder or free (Figures 2 and 3, Supplementary Table S6). Additional factors (run) as well as interactions, and covariates (male and female weight), for all other models except zig-zag for female free, and number of offspring, were non-significant (Supplementary Table S6).

Paternal care

Fanning duration increased from incubation day 2 to day 7, and decreased slightly on day 8 (Table 3, Figure 4). There was also a significant main effect of run (Table 3), with males in run 1 having a slightly higher fanning duration compared to males in run 2 (mean ± SE: run 1: 22.7 ± 0.86 s min⁻¹, run 2: 19.0 ± 1.26 s min⁻¹). There was no effect of CO₂ treatment or number of offspring, and all 2- and 3-way interactions had no significant effect (Supplementary Table S7).

Male sexual ornaments

The analyses of sexual ornaments showed that male weight had a significant effect on the intensity of the color of the ornaments (Table 4). Larger males had higher values for jaw *a* and *b*, meaning

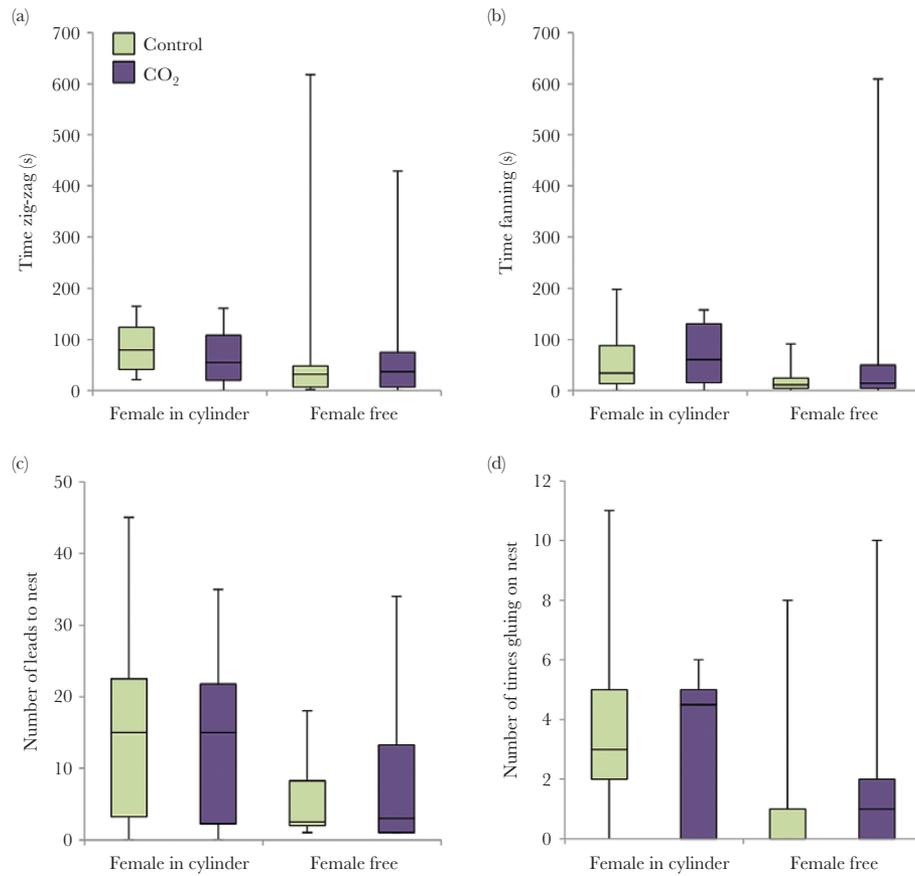


Figure 2

Time spent performing the courtship behaviors zig-zag (a) and fanning (b), and number of leads to the nest (c) and times gluing on nest (d). Control treatment in light green boxes and elevated CO₂ in dark purple. Behaviors recorded when the female was enclosed in the cylinder are to the left in each graph and recorded when the female was released (female free) are to the right in each graph. The boxplots show first and third quartiles (boxes), the median (line in box), and the minimum and maximum values (whiskers).

they had a more orange jaw (photo event 1, jaw *a*: $r = 0.51$, $t_{34} = 4.38$, $P < 0.001$, jaw *b*: $r = 0.43$, $t_{34} = 3.47$, $P = 0.001$; photo event 3 jaw *a*: $r = 0.64$, $t_{34} = 4.90$, $P < 0.001$, jaw *b*: $r = 0.49$, $t_{34} = 3.30$, $P = 0.002$), and lower values for iris *a*, meaning they had a more blue iris (photo event 1: $r = 0.47$, $t_{34} = -3.95$, $P < 0.001$, photo event 2: photo event 2: $r = 0.38$, $t_{34} = -3.02$, $P = 0.004$). There was also a significant effect of run for jaw *a* at photo event 1 (Table 4), due to slightly higher jaw *a* values for males from run 2 (mean \pm SE, run 1: 132.4 ± 1.56 , run 2: 133.7 ± 1.37), although this result may not be biologically relevant given the small difference. There was however no effect of the CO₂ treatment on any of the jaw or iris measurements, or on the interactions between run and treatment (Supplementary Table S8).

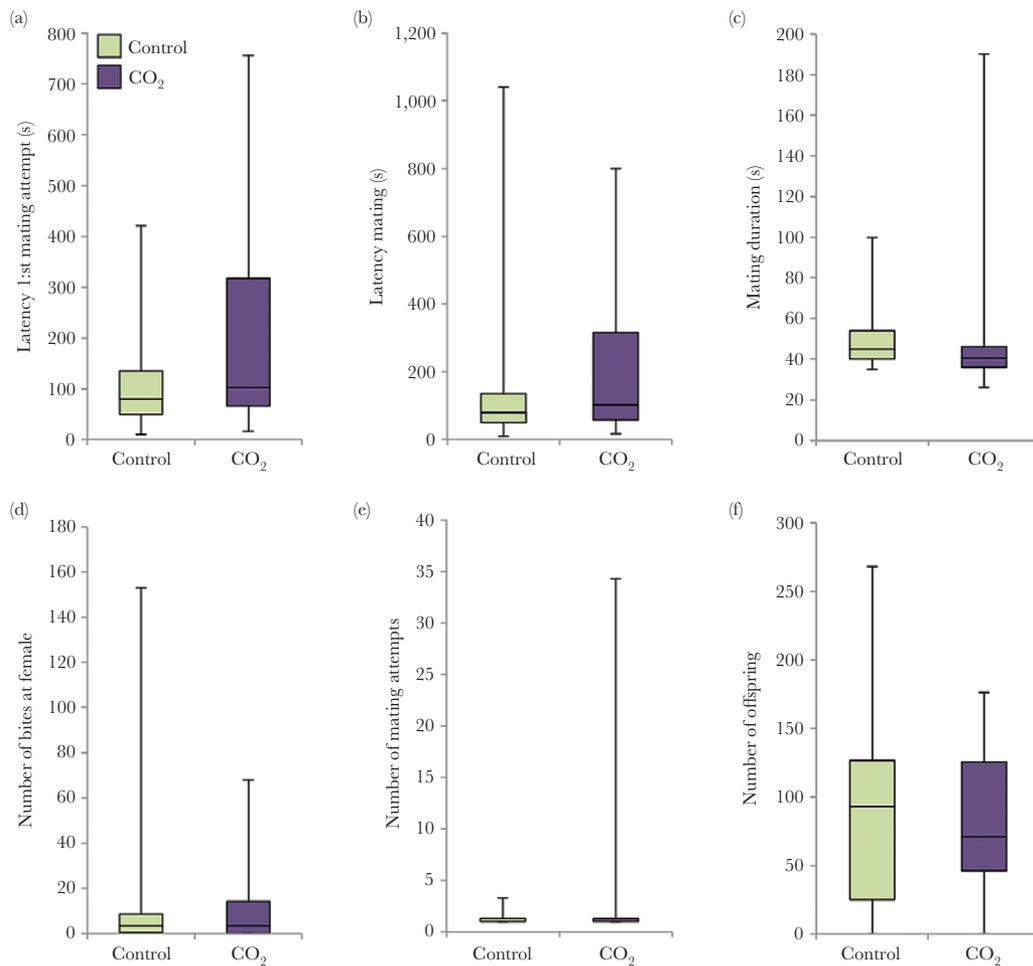
DISCUSSION

While many studies report detrimental effects of increased levels of dissolved carbon dioxide on behavior and physiology in fishes (reviewed in: Heuer and Grosell 2014; Clements and Hunt 2015), virtually nothing is known about the potential effect of CO₂ on fish reproduction. Here, we investigated the possible effects of elevated CO₂ on the complete breeding cycle. The results indicates that none of the reproductive variables were affected by the CO₂ treatment, suggesting that reproduction in the three-spined stickleback is likely to be largely unaffected by future elevated CO₂. We did however find positive effects of size for both males and females. Larger

females had a longer mating duration and provided males with more eggs, and larger males had more pronounced sexual ornaments. Moreover, fanning duration increased during the egg incubation period, hence, in sum, our results follow what is expected for this species (Milinski and Bakker 1990; Baker 1994; Fletcher and Wootton 1995), suggesting that the experimental design used was sound, and that CO₂ treatment effects, if present, would most likely have been detected. Our results constitute an important contribution to the CO₂ literature given that the possible impact of elevated levels of CO₂ on reproduction in fish has been largely ignored (Heuer and Grosell 2014; Milazzo et al. 2016).

Male sexual ornaments

Sexual ornaments are used to attract mates and to deter rivals, and are not only shaped by sexual selection but also natural selection, such as environmental conditions (Maynard Smith and Harper 2003). Although we are not aware of any studies investigating the possible effects of elevated CO₂ on sexual ornaments, other anthropogenic stressors, for example turbidity, agricultural runoff and sewage, have been reported to impose various effects (reviewed in: van der Sluijs et al. 2011). Living in the stressful environment that elevated CO₂ potentially constitutes could thus be expected to alter the development and/or display of sexual ornaments. The lack of a CO₂ effect on the blue iris and orange jaw however indicates that three-spine stickleback males cope well with elevated CO₂ water in

**Figure 3**

Latency until the first mating attempt (a), until the successful mating (b), and mating duration (c), as well as number of bites at female (d), number of mating attempts (e), and number of offspring (f). Control treatment in light green boxes and elevated CO₂ in dark purple. The boxplots show first and third quartiles (boxes), the median (line in box), and the minimum and maximum values (whiskers).

Table 3

The effect of treatment (high CO₂ and control), run (1 and 2), incubation day (day 2–8), number of offspring, and the interactions, on mean fanning duration ($n = 14$ males per treatment)

Response variable	Explanatory variable	SS	MS	DF	<i>F</i>	<i>P</i>
Egg fanning	Intercept			1.14	482.58	< 0.001
	Incubation day	3670.50	611.75	6.14	12.00	< 0.001
	Run	242.17	242.17	1.25	4.75	0.039

Sum of squares and mean squares are from a LMM with replicate (male ID) as a random factor. Only the minimum adequate model is presented (for full model, see Supplementary Table S7).

this respect. Although the relationship between intensity of nuptial coloration and physical condition is not always positive (Candolin 1999) in this species, indicating a great complexity of this trait (Svensson and Wong 2011), many studies report that male condition (and/or size) has an effect on male sexual ornaments (Maynard Smith and Harper 2003; Svensson and Wong 2011). This was also found here, where larger males had a bluer iris and a more orange jaw, hence our results confirmed what is expected for this species.

Precopulatory behaviors

The main purpose of a nest is obviously to function as a repository for eggs and developing offspring, but nest construction and

maintenance may also serve as an extended ornament in species where the nest is build solely by one sex, such as in the three-spined stickleback (Barber et al. 2001b; Östlund-Nilsson and Holmlund 2003). Whether elevated CO₂ affects nest building behavior has, to our knowledge, not been investigated. However, yet again this is a behavior that has previously been shown to be affected by other environmental stressors, such as endocrine disrupting chemicals (Saaristo et al. 2010). Most previous studies on the effects of CO₂ on reproduction have used species that lay their eggs on a substratum that can be mimicked in the lab using artificial material (e.g. a terracotta pot: Miller et al. 2013; Miller et al. 2015, or PVC tube: Forsgren et al. 2013), or have employed

artificial fertilization and rearing (Schade et al. 2014), thus not enabling any observations on nest construction or maintenance. The ocellated wrasse, used in (Milazzo et al. 2016), has a similar nest building behavior as the stickleback, and although nest building behavior was not explicitly investigated in that study, it may be assumed that CO₂ would have had little effects since reproductive output was not affected by the CO₂ treatment. In the three-spined stickleback, the nest is constructed by gluing together filamentous algae with Spiggin, a glycoprotein that is produced by the kidney and under the control of androgenic hormones (Jakobsson et al. 1999; Barber et al. 2001b). The male may also put more

Spiggin on the nest as part of the courtship ritual, herein referred to as “gluing” (Wootton 1976). Elevated CO₂ has been reported to increase the level of certain sex steroid hormones in Atlantic cod, *Gadus morhua*, (Preus-Olsen et al. 2014), hence it is conceivable that CO₂ could alter the production of Spiggin, if additional hormones are affected. Detailed hormone analysis will however be required to explicitly answer this question.

Courtship and mating

Courtship has many functions, for example to convey individual quality, ensure reproductive compatibility, prevent inbreeding, and reduce hostility between potential partners (Bateson 1983; Andersson 1994). Courtship and mating are sensitive to environmental change and may for example be altered by turbidity (reviewed in (Wong and Candolin 2015), hypoxia (reviewed in Pollock et al. 2007), agricultural contaminants (Saaristo et al. 2013; Bertram et al. 2015), and sewage runoff (Saaristo et al. 2014). Since we found no effects of elevated CO₂ on any of the courtship behaviors measured (e.g. zig-zag, displacement fanning, leads to the nest, gluing) or the variables measured for mating (latency until 1:st mating attempt, number of mating attempts, latency until mating, and mating duration), this again suggests that courtship and mating is not affected by this environmental stressor. Although there was great variation between individuals, the significant effect of male size on courtship intensity (zig-zag duration) as well as the greater number of eggs being produced from larger males and females follows what is expected in this species (Wootton 1973). This indicates that a treatment effect, if present, would most likely have been detected.

Similarly to the results on courtship and mating, we found no effect of the CO₂ treatment on number of offspring. In contrast, it has previously been reported that this species produce more eggs when kept at elevated levels of CO₂ (Schade et al. 2014). This discrepancy between studies is also found for the cinnamon anemone fish, *A. melanopus*, where one study report that breeding pairs kept in elevated CO₂ produced more eggs per clutch and more clutches per breeding pair (Miller et al. 2013), while a later study (on the same species) report essentially no effects of elevated CO₂ on the

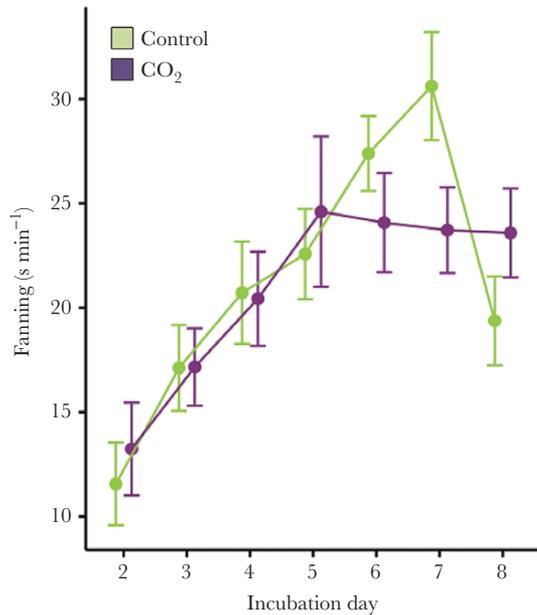


Figure 4 Changes in time spent fanning the nest over the nesting cycle (incubation day 2–8, with spawning taking place on incubation day 0) in the 2 treatment groups (light green: control, dark purple: elevated CO₂). Error bars show standard error.

Table 4 The effect of treatment (high CO₂ and control), run (1 and 2), and male weight on values from the image analysis (*a* and *b* for iris and jaw)

Photo event	Response variable	Explanatory variable	Estimate (± SE)	DF	χ ²	<i>P</i>
1	iris <i>a</i>	Intercept	4.81 (0.01)		249417.9	< 0.001
		Male weight	-0.03 (0.01)	1, 54	15.72	< 0.001
	jaw <i>a</i>	Intercept	4.76 (0.03)		34016.6	< 0.001
		Run	0.04 (0.01)	1, 53	7.99	0.005
		Male weight	0.08 (0.01)	1, 53	28.90	< 0.001
		Intercept	4.88 (0.04)		16526.3	< 0.001
jaw <i>b</i>	Male weight	0.09 (0.02)	1, 54	11.64	0.001	
	Intercept	4.82 (0.01)		136384.5	< 0.001	
2	iris <i>a</i>	Intercept	4.82 (0.01)		136384.5	< 0.001
		Male weight	-0.03 (0.01)	1, 54	9.22	0.002
	jaw <i>a</i>	Intercept	4.86 (0.02)		48735.6	< 0.001
		Male weight	0.04 (0.01)	1, 54	5.73	0.017
		Intercept	4.85 (0.02)		73369.2	< 0.001
		Male weight	-0.02 (0.04)	1, 34	10.43	0.001
jaw <i>a</i>	Intercept	4.91 (0.01)		30066.6	< 0.001	
	Male weight	0.09 (0.02)	1, 34	23.53	< 0.001	
	Intercept	4.86 (0.06)		7632.5	< 0.001	
	Male weight	0.24 (0.11)	1, 34	8.59	0.003	

a is green to red and *b* is blue to yellow. Each photo event (1–3) was analyzed separately. *n* = 28 males per treatment for photo event 1 and 2, *n* = 18 for photo event 3 (the males that mated). Only the minimum adequate models are presented (for full models, see Supplementary Table S8).

very same traits (Miller et al. 2015). This great discrepancy in results obtained when using the same species highlights the need for replication and shows the vulnerability of drawing general conclusions on the effects of elevated CO₂ based on few studies.

Paternal care

Fanning is a common parental activity in fish (Blumer 1979), and this behavior too is sensitive to environmental change and can be adjusted to changes in for example oxygen levels and temperature (Reebs et al. 1984; Torricelli et al. 1985). In the three-spined stickleback, brood-fanning is a very important part of paternal care, and it is energetically costly (reviewed in: Östlund-Nilsson 2007). Since the purpose of fanning is to oxygenate the eggs, it could be hypothesized that elevated levels of CO₂ should increase fanning as oxygen and CO₂ concentrations are often inversely related. We found that males increased their fanning duration as the nesting cycle progressed, peaking at incubation day 7 and declined at day 8, which is expected for this species (Páll et al. 2002). We did not detect an overall effect of CO₂ treatment on fanning duration. The males in run 1 had a slightly higher fanning duration compared to males in run 2, however the mean difference was relatively small. It is again difficult to put these results in context to other findings on the effects of elevated CO₂, since the previous studies either did not investigate fanning behavior (Milazzo et al. 2016, although the species do fan the nest), or employed an artificial rearing design (Schade et al. 2014), thus not incorporating the possible effects on parental care.

CONCLUSIONS

This study offers a broad investigation on the effects of elevated CO₂ on reproduction, an area of the CO₂ research where the scientific community repeatedly has highlighted a serious knowledge gap (Ishimatsu et al. 2008; Heuer and Grosell 2014; Milazzo et al. 2016). Our results follow what is expected for this species, in terms of larger males having more expressed sexual ornaments, larger females providing males with more eggs, and fanning duration increasing over the brooding period, confirming that the experimental design used was sound. We did not detect any effects of the elevated CO₂ treatment, indicating that the highly fitness-related functions of reproduction are robust to elevated CO₂. The results presented here therefore provide an optimistic view with regards to fish reproduction and future elevated levels of CO₂.

SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

FUNDING

This work was supported by the Swedish Research Council Formas (2013–947 to J.S. and 2009–596 to F.J.); the Swedish Research Council VR (621-2012-4679 to F.J.); and by the Royal Swedish Academy of Sciences (FOA14SLC027 to J.S. and FOA14SLC016 to L.V.).

We thank Johan Rudin and Arianna Cocco for field assistance. Thanks to Bengt Lundve for technical assistance, and to Ido Pen for statistical advice. The Sven Lovén Centre for Marine Infrastructure, Kristineberg, provided

excellent laboratory facilities. Thanks to 2 anonymous reviewers for constructive advice.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Sundin et al. (2017).

Authors' contributions: J.S., L.V., and F.J. designed the experiment; J.S., L.V., and F.J. collected the data; H.N.S. analyzed the images; J.S. and L.V. analyzed the data. J.S. led the writing of the manuscript. All authors contributed to the draft and gave final approval for publication.

Handling editor: John Fitzpatrick

REFERENCES

- Andersson M. 1994. *Sexual Selection*. Princeton: Princeton University Press.
- Baker JA. 1994. Life history variation in female threespine stickleback. In: Bell MA, Foster SA, editors. *The Evolutionary Biology of the Threespine Stickleback*. Oxford: Oxford University Press. p. 144–187.
- Barber I, Arnott SA, Braithwaite VA, Andrew J, Huntingford FA. 2001a. Indirect fitness consequences of mate choice in sticklebacks: offspring of brighter males grow slowly but resist parasitic infections. *Proc R Soc Lon B Biol*. 268:71–76.
- Barber I, Nairn D, Huntingford FA. 2001b. Nests as ornaments: revealing construction by male sticklebacks. *Behav Ecol*. 12:390–396.
- Barrett RD, Paccard A, Healy TM, Bergek S, Schulte PM, Schluter D, Rogers SM. 2011. Rapid evolution of cold tolerance in stickleback. *Proc R Soc Lon B Biol*. 278:233–238.
- Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *J Stat Softw*. 67:1–48.
- Bateson P. editor 1983. *Mate choice*. Cambridge: Cambridge University Press.
- Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. *J R Stat Soc Series B Stat Methodol*. 57:289–300.
- Bertram MG, Saaristo M, Baumgartner JB, Johnstone CP, Allinson M, Allinson G, Wong BBM. 2015. Sex in troubled waters: Widespread agricultural contaminant disrupts reproductive behaviour in fish. *Horm Behav*. 70:85–91.
- Blumer LS. 1979. Male parental care in the bony fishes. *Q Rev Biol*. 54:149–161.
- Browman HI. 2016. Applying organized scepticism to ocean acidification research. *ICES J Mar Sci*. 73:529–536.
- Caldeira K, Wickett ME. 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J Geophys Res-Oceans*. 110:1–12.
- Candolin U. 1997. Predation risk affects courtship and attractiveness of competing threespine stickleback males. *Behav Ecol Sociobiol*. 41:81–87.
- Candolin U. 1999. The relationship between signal quality and physical condition: is sexual signalling honest in the three-spined stickleback? *Anim Behav*. 58:1261–1267.
- Candolin U, Tukiainen I. 2015. The sexual selection paradigm: have we overlooked other mechanisms in the evolution of male ornaments? *Proc R Soc Lon B Biol*. 282:20151987.
- Clements JC, Hunt HL. 2015. Marine animal behaviour in a high CO₂ ocean. *Mar Ecol Prog Ser*. 536:259–279.
- Cornwall CE, Hurd CL. 2016. Experimental design in ocean acidification research: problems and solutions. *ICES J Mar Sci*. 73:572–581.
- Dupont S, Dorey N, Thorndyke M. 2010. What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? *Estuar Coast Shelf Sci*. 89:182–185.
- Flamarique IN, Bergstrom C, Cheng CL, Reimchen TE. 2013. Role of the iridescent eye in stickleback female mate choice. *J Exp Biol*. 216:2806–2812.
- Fletcher DA, Wootton RJ. 1995. A hierarchical response to differences in ration size in the reproductive-performance of female 3-spined sticklebacks. *J Fish Biol*. 46:657–668.
- Forsgren E, Dupont S, Jutfelt F, Amundsen T. 2013. Elevated CO₂ affects embryonic development and larval phototaxis in a temperate marine fish. *Ecol Evol*. 3:3637–3646.
- Frischknecht M. 1993. The breeding coloration of male 3-spined sticklebacks (*Gasterosteus aculeatus*) as an indicator of energy investment in vigor. *Evol Ecol*. 7:439–450.

- Heuer RM, Grosell M. 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *Am J Physiol-Reg I*. 307:R1061–R1084.
- Hurlbert SH. 1984. Pseudoreplication and the design of ecological field experiments. *Ecol Monogr*. 54:187–211.
- Ishimatsu A, Hayashi M, Kikkawa T. 2008. Fishes in high-CO₂, acidified oceans. *Mar Ecol Prog Ser*. 373:295–302.
- Jakobsson S, Borg B, Haux C, Hyllner S. 1999. An 11-ketotestosterone induced kidney-secreted protein: the nest building glue from male three-spined stickleback, *Gasterosteus aculeatus*. *Fish Physiol Biochem*. 20:79–85.
- Jutfelt F, Bresolin de Souza K, Vuylsteke A, Sturve J. 2013. Behavioural disturbances in a temperate fish exposed to sustained high-CO₂ levels. *PLoS ONE*. 8:e65825.
- Jutfelt F, Hedgärde M. 2013. Atlantic cod actively avoid CO₂ and predator odour, even after long-term CO₂ exposure. *Front Zool*. 10:81.
- Kraak SBM, Bakker TCM, Mundwiler B. 1999. Sexual selection in sticklebacks in the field: correlates of reproductive, mating, and paternal success. *Behav Ecol*. 10:696–706.
- Kuznetsova A, Brockhoff PB, Bojesen-Christensen RH. 2016. Tests in linear mixed effects model. R package version 2.0 [cited 2016 December 3]. Available from: <https://cran.r-project.org/web/packages/lmerTest/index.html>.
- Lai F, Jutfelt F, Nilsson GE. 2015. Altered neurotransmitter function in CO₂-exposed stickleback (*Gasterosteus aculeatus*): a temperate model species for ocean acidification research. *Conserv Physiol*. 3:cov018.
- Mayer I, Páll M. 2007. Hormonal control of reproductive behaviour in the stickleback. In: Östlund-Nilsson S, Mayer I, Huntingford FA, editors. *Biology of the three-spined stickleback*. Boca Raton: Taylor & Francis Group. p. 249–269.
- Maynard Smith J, Harper D. 2003. *Animal Signals*. Oxford: Oxford University Press.
- Melzner F, Gutowska MA, Langenbuch M, Dupont S, Lucassen M, Thorndyke MC, Bleich M, Pörtner HO. 2009. Physiological basis for high CO₂ tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences*. 6: 2313–2331.
- Milazzo M, Cattano C, Alonzo SH, Foggo A, Gristina M, Rodolfo-Metalpa R, Sinopoli M, Spatafora D, Stiver KA, et al. 2016. Ocean acidification affects fish spawning but not paternity at CO₂ seeps. *Proc R Soc Lon B Biol*. 283:20161021.
- Milinski M, Bakker TCM. 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature*. 344:330–333.
- Miller GM, Kroon FJ, Metcalfe S, Munday PL. 2015. Temperature is the evil twin: effects of increased temperature and ocean acidification on reproduction in a reef fish. *Ecol Appl*. 25:603–620.
- Miller GM, Watson S-A, McCormick MI, Munday PL. 2013. Increased CO₂ stimulates reproduction in a coral reef fish. *Glob Change Biol*. 19:3037–3045.
- Näslund J, Lindström E, Lai F, Jutfelt F. 2015. Behavioural responses to simulated bird attacks in marine three-spined sticklebacks after exposure to high CO₂ levels. *Mar Freshw Res*. 66:877–885.
- Östlund-Nilsson S. 2007. Reproductive behaviour in the three-spined stickleback. In: Östlund-Nilsson S, Mayer I, Huntingford FA, editors. *Biology of the three-spined stickleback*. Boca Raton: Taylor & Francis Group. p. 157–176.
- Östlund-Nilsson S, Holmlund M. 2003. The artistic three-spined stickleback (*Gasterosteus aculeatus*). *Behav Ecol Sociobiol*. 53:214–220.
- Páll MK, Mayer I, Borg B. 2002. Androgen and Behavior in the male three-spined stickleback, *Gasterosteus aculeatus*: II. Castration and 11-ketotestosterone effects on courtship and parental care during the nesting cycle. *Horm Behav*. 42:337–344.
- Pollock MS, Clarke LMJ, Dube MG. 2007. The effects of hypoxia on fishes: from ecological relevance to physiological effects. *Environ Rev*. 15:1–14.
- Pottinger T, Carrick T, Yeomans W. 2002. The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *J Fish Biol*. 61:207–229.
- Preus-Olsen G, Olufsen MO, Pedersen SA, Letcher RJ, Arukwe A. 2014. Effects of elevated dissolved carbon dioxide and perfluorooctane sulfonic acid, given singly and in combination, on steroidogenic and biotransformation pathways of Atlantic cod. *Aqua Tox*. 155:222–235.
- R Core Team. 2013. R: A language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing. Available from: <http://www.R-project.org/>.
- Rees S, Whoriskey Jr F, FitzGerald G. 1984. Diel patterns of fanning activity, egg respiration, and the nocturnal behavior of male three-spined sticklebacks, *Gasterosteus aculeatus* L. (f. trachurus). *Can J Zool*. 62:329–334.
- Saaristo M, Craft JA, Lehtonen KK, Lindström K. 2010. Exposure to 17 alpha-ethinyl estradiol impairs courtship and aggressive behaviour of male sand gobies (*Pomatoschistus minutus*). *Chemosphere*. 79:541–546.
- Saaristo M, Myers J, Jacques-Hamilton R, Allinson M, Yamamoto A, Allinson G, Pettigrove V, Wong BBM. 2014. Altered reproductive behaviours in male mosquitofish living downstream from a sewage treatment plant. *Aqua Tox*. 149:58–64.
- Saaristo M, Tomkins P, Allinson M, Allinson G, Wong BBM. 2013. An androgenic agricultural contaminant impairs female reproductive behaviour in a freshwater fish. *Plos One*. 8:e62782.
- Schade FM, Clemmesen C, Wegner KM. 2014. Within- and transgenerational effects of ocean acidification on life history of marine three-spined stickleback (*Gasterosteus aculeatus*). *Mar Biol*. 161:1667–1676.
- Sundin J, Jutfelt F. 2016. 9–28 d of exposure to elevated pCO₂ reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (*Ctenolabrus rupestris*). *ICES J Mar Sci*. 73:620–632.
- Sundin J, Rosenqvist G, Berglund A. 2013. Altered oceanic pH impairs mating propensity in a pipefish. *Ethology*. 119:86–93.
- Sundin J, Vossen L, Nilsson-Sköld H, Jutfelt F. 2017. Data from: no effect of elevated carbon dioxide on reproductive behaviors in the three-spined stickleback. Dryad Digital Repository. <http://dx.doi.org/10.5061/dryad.n99n3>.
- Svensson PA, Forsgren E, Amundsen T, Sköld HN. 2005. Chromatic interaction between egg pigmentation and skin chromatophores in the nuptial coloration of female two-spotted gobies. *J Exp Biol*. 208:4391–4397.
- Svensson PA, Wong BBM. 2011. Carotenoid-based signals in behavioural ecology: a review. *Behaviour*. 148:131–189.
- Torricelli P, Lugli M, Gandolfi G. 1985. A quantitative analysis of the fanning activity in the male *Padogobius martensi* (Pisces: Gobiidae). *Behaviour*. 92:288–301.
- van der Sluijs I, Gray SM, Amorim MCP, Barber I, Candolin U, Hendry AP, Krahe R, Maan ME, Utne-Palm AC, Wagner H-J, et al. 2011. Communication in troubled waters: responses of fish communication systems to changing environments. *Evol Ecol*. 25:623–640.
- Wong BBM, Candolin U. 2015. Behavioral responses to changing environments. *Behav Ecol*. 26:665–673.
- Wootton R. 1973. The effect of size of food ration on egg production in the female three-spined stickleback, *Gasterosteus aculeatus* L. *J Fish Biol*. 5:89–96.
- Wootton R. 1976. *The biology of the sticklebacks*. London: Academic Press.
- Zuur A, Ieno E, Walker N, Saveliev A, Smith G. 2009. *Mixed effects models and extensions in ecology with R*. New York (NY): Springer Science and Business Media.