Title: Temperature is the evil twin: Effects of increased temperature and ocean acidification on reproduction in a reef fish.

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Abstract

Reproduction in many organisms can be disrupted by changes to the physical environment, such as those predicted to occur during climate change. Marine organisms face the dual climate change threats of increasing temperature and ocean acidification, yet no studies have examined the potential interactive effects of these stressors on reproduction in marine fishes. We used a long-term experiment to test the interactive effects of increased temperature and CO_2 on the reproductive performance of the anemonefish, *Amphiprion melanopus*. Adult breeding pairs were kept for 10 months at three temperatures, 28.5°C (+0.0°C), 30.0°C (+1.5°C) and 31.5°C (+3.0°C), cross-factored with 3 CO_2 levels, a current day control (417µatm) and moderate (644µatm) and high (1134µatm) treatments consistent with the range of CO_2 projections for the year 2100 under RCP8.5. We recorded each egg clutch produced during the breeding season, the number of eggs laid per clutch, average egg size, fertilization success, survival to hatching, hatchling length and yolk provisioning. Adult body condition, hepatosomatic index, gonadosomatic index, and plasma 17β-estradiol concentrations were measured at the end of the breeding season to determine the effect of prolonged exposure to increased temperature and elevated CO_2 on adults, and to examine potential physiological mechanisms for changes in reproduction. Temperature had by far the stronger influence on reproduction, with clear declines in reproduction occurring in the +1.5°C treatment and ceasing altogether in the +3.0°C treatment. In contrast, CO_2 had a minimal effect on the majority of reproductive traits measured, but caused a decline in offspring quality in combination with elevated temperature. We detected no significant effect of temperature or CO_2 on adult body condition or hepatosomatic index. Elevated temperature had a significant negative effect on plasma 17β-estradiol concentrations, suggesting that declines in reproduction with increasing temperature were due to the thermal sensitivity of reproductive hormones rather than a reduction in energy available for reproduction. Our results show that elevated temperature exerts a stronger influence than high CO_2 on reproduction in *A. melanopus*. Understanding how these two environmental variables interact to affect the reproductive performance of marine organisms will be important for predicting the future impacts of climate change.

Keywords: Reproduction, ocean acidification, carbon dioxide, ocean warming, *Amphiprion melanopus*, climate change

Introduction

Reproduction is critical to individual fitness and the persistence of populations. Reproduction in most organisms is also sensitive to changes in the physical environment. For example, the timing of reproduction can be influenced by variation in temperature (Kjesbu, 1994; Visser *et al.,* 2009), photoperiod (Duston & Bromage, 1986; Dawson *et al.*, 2001), rainfall (Donnelly & Guyer, 1994; Hau *et al.,* 2004) and flow regimes (Schlosser, 1982; Bunn & Arthington, 2002). Similarly, reproductive output is affected by temperature (King *et al*., 2003; Saino *et al*., 2004) and food availability (Brown & Shine, 2007; Donelson *et al*., 2010). Consequently, anthropogenic climate change is predicted to affect reproductive success of many species (Parmesan, 2006; Poloczanska *et al*., 2013) and could be the primary driver of population declines due to climate change (Van Der Kraak & Pankhurst, 1997; Zeh *et al.*, 2012).

For marine organisms, increasing temperature and ocean acidification are the most serious climate change threats (Hoegh-Guldberg *et al.,* 2007; Doney *et al.,* 2009) and they are predicted to be additive or synergistic in their effect on performance, potentially leading to greater effects in combination than in isolation (Pörtner & Farrel, 2008). Many studies have examined the effects of one or other of these two stressors on reproduction in marine organisms (including Miller *et al.*, 2013; Donelson *et al.,* 2010), but few have examined the potential interactive effects on reproduction. While an increasing number of studies are testing the interacting effects of ocean warming and ocean acidification on invertebrates (Byrne *et al.,* 2009, 2010; Parker *et al.,* 2009; Albright & Mason 2013, Cohen-Rengifo *et al*., 2013) relatively few studies have tested these two stressors in combination for fish (but see Munday *et al.,* 2009a; Nowicki *et al*., 2012; Grans *et al.,* 2014) and none have tested the interacting effects on reproduction in fishes. In fishes, increased temperature has been shown to negatively affect reproduction in many species (see reviews, Pankhurst & Porter, 2003;

Pankhurst & King, 2010). In contrast, ocean acidification, while predicted to have negative impacts (Pörtner & Farrel, 2008; Ishimatsu *et al*., 2008) has been found to have little impact, or even positive effects, on reproduction in multiple species of fish (Frommel *et al*., 2010; Sundin *et al.,* 2012, Miller *et al*., 2013; Fosgren *et al*., 2013). Yet, whether elevated temperature and ocean acidification will interact to affect reproduction in fishes is not known.

For many species of fish, temperature is one of the main cues for reproduction, signaling the beginning and the end of the breeding season (Van Der Kraak & Pankhurst, 1997; Pankhurst & Munday, 2011). For spring-summer spawner's, the increase in water temperatures following the winter minimum, elicits physiological changes, production of sex steroids, maturation of gonads, and spawning (Kjesbu, 1994; Pankhurst *et al*., 1996). Nevertheless, reproduction only occurs within a narrow range of temperatures that the population normally experiences (Van der Kraak & Pankhurst, 1997). If temperatures exceed this thermal window, reproduction can quickly decline and may cease altogether (Donelson *et al*., 2010; Dorts *et al.*, 2011). Tropical fishes may be especially sensitive to changes in temperature, as they inhabit a more thermally stable environment than higher latitude species (Tewksbury *et al.,* 2008; Rummer *et al.,* 2014). This means that even a relatively small increase in average temperature, such as predicted by climate change, could have serious effects on reproductive performance in tropical species (Donelson *et al.,* 2010; Zeh *et al*., 2012).

Reproduction may decline at elevated temperatures as a result of energetic constraints or through the effects of temperature on hormonal pathways. As temperatures increase past the thermal optimum, individuals need to expend more energy maintaining cellular function (Pörtner & Farrell, 2008). Organisms have a finite amount of energy available and as more of the energy is used for homeostasis less is available for other activities, such as reproduction

(Somero, 2002; Sokolova *et al.,* 2012). Under energy constraints, adults could opt to produce the same number of offspring as under normal conditions, but at a cost to offspring provisioning. Alternatively, individuals may produce fewer offspring that have adequate levels of provisioning in an attempt to ensure offspring survival (Stearns, 1992).

Reproduction may also decline with increasing temperature due to the thermal sensitivity of reproductive hormones. Reproduction in fish is tightly controlled through the interplay of multiple hormones and steroids created by the hypothalamus, the pituitary and the gonads (Hypothalamic-Pituitary-Gonadal axis (HPG axis)) (Yaron & Levavi-Sivan, 2011). Elevated temperatures have the ability to inhibit the HPG axis at multiple sites, through changes in hormone synthesis, action and structures (Pankhurst & Munday, 2011). The inhibitory effects of temperature can occur through changes in protein and hormone structures, resulting in a reduced uptake or insolubility of the hormones. These changes can then lead to the hormones failing to reach the correct receptor, or passing straight through the kidneys and being excreted, thereby impairing the particular reproductive process (Van Der Kraak & Pankhurst, 1997; Pankhurst & Munday, 2011). Ultimately, wherever the disruption to the hormonal cascade occurs, elevated temperatures result in declines in reproductive activity, egg size and offspring survival.

In addition to increasing temperatures, marine fishes will have to cope with increasing partial pressure of carbon dioxide (pCO_2) in the ocean. Increasing pCO_2 has been documented to negatively impact reproduction in a number of invertebrates (see Ross *et al.,* 2011). Fishes, however, have well-developed mechanisms for acid-base regulation and are able to maintain their internal pH against an elevated CO_2 gradient, through active transport of ions across the gills and in their blood and tissues (Brauner & Baker, 2009; Esbaugh *et al.,* 2012). This process is not cost free and it has been predicted that the increase in energy required to maintain acid-base balance should result in a decline in energy available for reproduction and other activities (Pörtner *et al.,* 2004; Ishimatsu *et al*., 2008). However, only one study (Inaba *et al.,* 2003) has documented a negative impact of increasing pCO_2 on a reproductive trait, with sperm motility being reduced in some flatfishes, but not in a range of other species. Other studies have reported little to no effects of increased CO_2 on reproduction. For example, Frommel *et al.* (2010) found no effect on sperm motility in Baltic cod (*Gadhus morhua*), Sundin *et al.* (2012) found no difference in reproductive propensity in pipefish (*Syngnathus typhle*) and Fosgren *et al.* (2013) found no differences in clutch size but did see a significant decline in egg survival with increasing CO_2 in a temperate goby (*Gobiusculus flavescens*). Interestingly, several studies have documented increases in reproduction or reproductive related traits in response to elevated CO_2 (Miller *et al*., 2013; Schade *et al.,* 2014) Furthermore, Preus-Olsen *et al.* (2014) documented increased levels of sex steroid hormones in Atlantic cod at high CO_2 which is consistent with greater rates of reproduction in the other studies. Neither Miller *et al.* (2013) or Schade *et al.* (2014) found negative consequences of the increased reproductive activity in high CO_2 on the condition of the adults or the resulting offspring. Instead these studies show transgenerational acclimation of the offspring to elevated CO_2 due to parental exposure to high CO_2 (Miller *et al.,* 2012: Schade *et al.,* 2014).

Increases in temperature and pCO_2 will not occur in isolation from each other, and for that reason, it is important to understand how they may interact to affect reproduction. As these two variables have been documented to have contrasting effects on reproduction in fish, it is especially important to understand how they might interact to affect this critical process. The aim of this study was to document the effect of elevated CO_2 and increased temperature on reproductive activity, offspring quality, and any effect on adult condition (physical and reproductive) in a tropical reef fish. Adult pairs of *Amphiprion melanopus* were kept in current-day control CO_2 or elevated CO_2 treatments (moderate and high). CO_2 treatments were fully cross-factored with 3 temperature treatments, current-day summer average water temperature, 28.5°C (+0.0°C), or two elevated temperatures, 30.0°C (+1.5°C) and 31.5°C (+3.0°C). Adult pairs were placed in CO_2 treatment during winter, slowly brought up to the required temperature treatments through spring and then allowed to reproduce naturally during the summer breeding season. Throughout the breeding season we assessed the effect of elevated temperature and increased CO_2 on key reproductive traits related to breeding and spawning, egg production and survival, and offspring provisioning (Fig. 1). At the end of the reproductive season we assessed adult physiological (Fulton's K body condition index, hepatosomatic index) and reproductive condition (gonadosomatic index, plasma hormone concentration) (Fig. 1) to determine if difference in reproductive performance were potentially associated with the energetic cost of reproduction or effects on reproductive hormones. 17β-estradiol (E_2) was chosen as the focal sex steroid due to its well-defined role in vitellogenesis and oocyte maturation in female fish (Lubzen *et al.,* 2010; Yaron & Levavi-Sivan, 2011).

Methods

Study species and husbandry

The cinnamon anemonefish, *Amphiprion melanopus* (Pomacentridae) inhabits coral reefs throughout the Indo-Pacific region, including the Great Barrier Reef, Australia (Drew *et al.,* 2008). *Amphiprion melanopus* occur in large social groups containing multiple breeding pairs that reproduce repeatedly during the summer. Eggs are laid in clutches attached to hard substratum near their host anemone (Michael, 2008). Embryonic duration is 7-9 days, during which time the males tend the eggs. Larvae hatch after dark and are pelagic for approximately 11 days, at which point they metamorphose and become competent to settle to reef habitat (Bay *et al*., 2006). Adults reach a maximum length of 12cm (Lieske & Myers, 1994) and have a reported maximum age of 5 years (Allen, 1975).

Adult breeding pairs of *A. melanopus* were collected between June 2009 and June 2011 from 4 reefs in the central Great Barrier Reef: Orpheus Island (18.6183°S, 146.4936°E), Bramble Reef (18.417°S, 146.700°E), Davies Reef (18.83°S, 147.63°E) and Slasher's Reef (18.467°S, 147.083°E) and transferred to James Cook University, Townsville. Pairs were housed individually in 45L aquaria and provided with a half terracotta post as a nest site and shelter. Aquaria were provided with continuous flow of seawater at 1.5Lmin^{-1}. Pairs were fed 0.1g of commercial fish feed (INVE NRD 12/24) three times a day, equivalent to 1.21% of the average body weight (Donelson *et al.,* 2010).

Experimental design

Between seven and eight adult pairs of *A. melanopus* were assigned to each of the nine treatment groups and held individually, i.e. one pair per aquaria. Three CO_2 groups were cross-factored with three temperature groups reflective of pCO_2 and temperatures projected to occur in the ocean by 2100 under RCP6 and RCP8.5 (Collins *et al.,* 2013). The three CO_2 levels used were a current-day control (~417µatm), a moderate (~644 µatm) and a high (~1134 µatm) CO_2 treatment. The temperatures were the current-day summer average of the collection region, 28.5°C (+0.0°C), a moderate 30.0°C (+1.5°C) and a high, 31.5°C (+3.0°C) temperature treatments (See Table 1 for full experimental parameters). These temperatures reflect the current summer average water temperature for the Orpheus Island region where the adults were collected, and the 1.5 to 3.0°C warming predicted to occur in the tropical oceans over the coming century due to climate change (Poloczanska *et al.,* 2007; Ganachaud *et al.,* 2011).

Three 8000L liter aquarium systems were utilized, each dosed to the desired pCO_2. Due to the scale of the study it was not possible to have separate CO_2 and temperature treatments applied to each aquarium. Pairs were in their respective CO_2 treatments by June 2011 at current day winter water temperatures (22.5°C). Temperatures were subsequently increased over a two month period to achieve the required temperature separation and then increased by 0.5°C weekly to reach experimental breeding temperatures in the second week of November 2011.

Data collection of key reproductive traits

Throughout the breeding season, key reproductive traits related to reproductive activity, egg survival, offspring provisioning and adult physiological and reproductive condition were collected (Fig. 1). Terracotta pots were checked daily between 0900 and 1100 for the presence of egg clutches. A digital photograph (Canon G12) was taken of each new clutch. A sample of 10-20 eggs was then taken from the clutch and preserved in 6% formalin. Daily photographs of each clutch were taken until the eggs hatched or there were no more eggs due to mortality. Parents often eat the eggs if development is abnormal or if eggs are diseased. Clutches were considered successful if any eggs survived to 6 – 8 days post-spawning. Surviving egg clutches were hatched into 70L aquaria at the same pCO_2 and temperature treatment as their parents. A sample of between 10-20 larvae was taken the morning after hatching between 0730 and 0830, within 12 hours of hatching. Larvae were euthanized with an overdose of clove oil before being preserved in 4% phosphate buffered formaldehyde. A digital photograph (Canon G12) of each larva was taken on a 5mm grid in a horizontal position within 3 days of sampling.

The number of eggs laid in each clutch and the number of eggs remaining at hatching was counted from the digital image with the aid of ImageJ. The percentage of surviving eggs was then determined from these two counts. Fertilization success was determined by counting the number of unfertilized eggs in the initial clutch photograph. Unfertilized eggs were identified by their white colouration, whereas fertilized eggs had an orange colouration. To determine egg size the eggs sampled from each clutch were photographed (Canon G12) while placed horizontally on a 5mm grid so that the longest axis was visible. The image was viewed on a computer screen and ImageJ was used to trace the outside of 5 eggs from each sample and the average egg area (mm^2) was determined. Reproductive output for each clutch was estimated by multiplying the total number of eggs by the average egg area for that clutch providing a relative estimate of investment for each clutch (mm^2). Hatchling standard length (SL) was measured to the nearest 0.1mm from a photograph using ImageJ, by drawing a line from the tip of the mouth to the beginning of the tail. Yolk area was determined to the nearest $0.1mm^2$ by tracing the yolk sac in ImageJ from photographs viewed on a computer screen.

Data collection of adult physiological and reproductive traits

Adults were euthanized at the end of the breeding season to examine the effects of increased temperature and pCO_2 on body condition, liver condition, oocyte production and gonadal steroidogenesis (17β-estradiol, E_2). Each fish was weighed (wet weight W, nearest 0.01g) and measured (standard length SL, to the nearest 0.01mm). Fulton's K body condition factor (body condition) was then calculated using the formula $K=100*(W/SL^3)$, where W is wet weight in grams and SL is standard length in centimeters. To maintain genetic material and allow for potential genetic analysis, liver and gonads were dissected and snap frozen in liquid nitrogen. After freezing they were weighed to the nearest 0.0001g and then fixed in 4% phosphate buffered formaldehyde for several days before storage in 100% ethanol. Hepatosomatic index and gonadosomatic index were determined by the formula HSI/GSI= (liver weight or gonad weight (g)/fish weight (g))*100.

Plasma 17β-estradiol quantification

Blood samples were taken from females prior to euthanasia to estimate 17β-estradiol (E_2) concentrations. 17β-estradiol was chosen due to its role in vitellogenesis and oocyte maturation in female fish. Thus, changes in E_2 concentration could result in changes yolk provisioning, egg size, the number of eggs per clutch, the number of clutches, hatchling survival and hatchling length (Lubzens *et al.,* 2010; Yaron & Levavi-Sivan, 2011). Changes in E_2 concentration can therefore provide a direct endocrine pathway to any changes in reproductive output.

Blood samples were taken towards the end of the breeding season to allow for a direct comparison of plasma hormone concentrations to the reproductive activity of the individual female. Each fish was caught using a hand-net and a cranial concussion was delivered to render the fish unconscious. Blood samples were taken immediately via caudal puncture using pre-heparinized syringes and centrifuged immediately after collection. Plasma was aspirated from the top of the sample and snap frozen in liquid nitrogen before being stored at -80°C until steroid measurement.

To determine the E_2 concentration in the plasma samples, a known volume of plasma was combined with a solvent (containing 1:1 ethyl acetate and N-hexane) in a glass test tube (1:4

plasma:solvent). The mixture was vortexed twice for 10 seconds, with the layers allowed to separate between each round. The upper clear liquid was then transferred into a clean glass test vial, and the extraction steps were repeated three times for each sample. The residual solvent mix was evaporated by heating to 37°C under a gentle stream of nitrogen. The extracted samples were then capped and frozen at -20°C until analysis.

Plasma concentrations of E_2 were measured using enzyme immunoassay (EIA) kits obtained from Cayman Chemicals, Sapphire Bioscience (No 582251), and validated for *A. melanopus* (S. Metcalfe, unpublished data). The manufacturers instructions were followed, except that extracted plasma samples were measured in duplicate. Absorbance at 405nm was measured on a spectophotomere (Thermo Multiskan Ascent). Average extraction efficiency was 114±5.1% (standard error) (n=4), intra-assay variability was 5.3% (n=7) and 13.7% (n=6), and inter-assay variability was 9.6% (n=13).

Liver and gonad samples

Fixed ovaries were embedded in histoparaffin and 5-µm sections were taken at 3 points along the longest axis. Sections were mounted on a glass slide and stained with Mayer's alum haematoxylin and Young's eosin-erythrosine. To determine the reproductive status of individuals, a transect was run along each representative section and the type of sex cell under 100-graticules marked on an eyepiece micrometer was recorded at a 10 times magnification. Female cells were categorized into: oogonia (Stage 1), perinucleolus (Stage 2), cortical alveolus (Stage 3), early vitellogenic oocytes (Stage 4) and late vitellogenic oocytes (Stage 5), following Genten *et al*. (2009). The relative abundance of each cell type in each section was calculated.

Aquarium systems and seawater analysis

The pCO_2 in each system was controlled by an AquaMedic AT-controller that dosed a 3000L sump with CO_2 to maintain the pH at the appropriate level for the desired pCO_2. The control temperature (+0.0°C) was maintained by circulating seawater through a SolarWise heater/chiller on each system. The +0.0°C temperature seawater was either delivered directly to the aquaria, or was sent through Toyesi inline 2.5kW heaters to raise the temperature +1.5°C or +3.0°C, prior to delivery to the aquaria.

pH_{NBS} (Hach HQ40d) and temperature (Comark C26 thermometer) were recorded daily from replicate aquariums for each treatment. Total alkalinity was estimated weekly by Gran Titration (Metrohm 888 Titrando titrator) and validated against certified reference material (A.G. Dickson Scipss Institute of Oceanography). Salinity (Hach HQ15d) was measured weekly. The Aqua Medic pH set points were adjusted as needed to maintain the desired pCO_2 on each system.

pCO_2 was calculated in CO2SYS (http://cdiac.ornl.gov/oceans/co2rprt.html) using the daily pH_{NBS} and temperature (°C) readings and the weekly total alkalinity and salinity measurements. As temperature affects seawater pCO_2, CO_2 levels were not exactly the same among temperature treatments within each CO_2 treatment group. Nevertheless, CO_2 treatments remained well separated and for simplicity, the average CO_2 levels are reported (417µatm, 644µatm and 1134µatm) and are referred to as control, moderate or high CO_2 treatments.

Data analysis

ANCOVA was used to compare the number of clutches produced per pair in each treatment group. The number of clutches produced was the dependent variable, CO_2 and temperature treatments were the fixed variables, and female weight the covariate.

Linear mixed effects models (LME) (Pinheiro & Bates, 2000) were used to analyze the reproductive characteristics: interclutch interval, average number of eggs laid per clutch, average fertilization success, average egg area and reproductive output per clutch. As the experiment aimed to determine the interactive effect of increased temperature and CO_2 on reproduction, only clutches that were produced after experimental temperatures were attained were included in the analysis. The simplest LME constructed included the reproductive characteristic of interest as the dependent variable, CO_2 and temperature treatments as the fixed variables, and female weight was included as a random variable because reproductive traits can be strongly weight-dependent in fishes (Model A). Model B was constructed as above, but also grouped the data according to the breeding pair. Model C added the step of allowing for heterogeneity of variance within each pair, as pairs will have naturally fluctuating reproductive effort. The model that best represented the data set was determined by comparing the Akaike Information Criterion. Hatchling length and yolk area were also analyzed using linear mixed effects models. These analyses were constructed in the same order as described above (Model A, Model B and Model C). For hatchling length and yolk area, data was grouped according to clutch ID and heterogeneity of variance was allowed to occur within each clutch, as this was the level of replication.

The proportion of clutches that survived to hatching, and the proportion of eggs within each clutch that survived to hatching, were analysed with a penalized quasi-likelihood general linear mixed model (Splus Mass library). Temperature and CO_2 treatment were fixed variables, and breeding pair was a random variable in each model. For clutch survival, the proportion of the number of clutches that survived was weighted against the number of clutches that were produced by each pair. All mixed effects models were constructed and compared in Splus.

Fixed factor ANOVA (Type III) was used to compare Fulton's K, hepatosomatic index, gonadosomatic index and plasma E_2 concentration of females. The physiological trait was the dependent variable and CO_2 and temperature treatment were the fixed variables. Where a significant difference was detected a Fisher's LSD test was used to determine which treatment groups were significantly different.

The relationship between plasma E_2 concentration and the stage of gonadal development in individual fish was examined using factor analysis (Manly 1994, Kroon *et al*., 2003). The data was transformed using a varimax raw rotation to differentiate the original variables by extracted factor. Initial analysis identified two factors, and these two factors were used as the independent variables in a multiple regression analysis, where the plasma concentration of E_2 was the dependent variable.

Results

Reproductive characteristics

Reproduction in all treatment groups began in early October 2011, a month prior to summer breeding temperatures being achieved. Reproduction continued throughout the breeding season at +0.0°C for all CO_2 treatments (Fig. 2a). In contrast, reproduction in the +3.0°C treatment groups, irrespective of CO_2 level, and in moderate CO_2 +1.5°C, effectively ceased within a month of experimental temperatures being attained (Fig. 2b,c). Reproduction in all other groups continued from late September 2011 through to mid April 2012 with no obvious peaks in reproductive activity (Fig. 2a,b). An unequal number of pairs reproduced in the treatment groups, with 5 pairs reproducing in the moderate +0.0°C but only 2 reproducing in the moderate +3.0°C (Fig 2). The total number of egg clutches produced was greatest at +0.0°C (N=152) and declined markedly with increasing temperature (N=53 at +1.5°C and N=8 at +3.0°C) (Fig. 2). At +0.0°C the moderate and high CO_2 groups produced more clutches in total compared to the control CO_2 group (n= 55, 58 and 39 respectively), but this trend was not apparent at higher temperatures. Temperature appeared to have a stronger effect on the moderate CO_2 breeding pairs, as the moderate +1.5°C did not reproduce successfully once temperatures were attained, whereas, the control and high CO_2 at the same temperature continued to reproduce successfully (Fig. 2b).

The average number of clutches produced per pair declined with increasing temperature (Table 2, Fig. 3a). At +3.0°C there was a decline of between 78% to 87% in the average number of clutches produced per pair in all CO_2 treatments compared to the respective +0.0°C clutches produced per pair (Fig. 3a). In contrast, elevated CO_2 had no significant effect on the number of clutches produced per pair (Table 2, Fig. 3a). There was no effect of female weight on the average number of clutches produced per pair (Table 2.)

Unsurprisingly, given the differences in the number of clutches produced among treatments, both temperature and elevated CO_2 significantly increased the interclutch interval (Table 3). This was most marked in the moderate CO_2 group, where interclutch interval increased from 15±0.8 (SE) days at +0.0°C to 96.5±115 (SE) days at +3.0°C. A similar, though less marked effect, was seen in the control CO_2 group where interclutch interval increased from 18±0.8 at +0.0°C to 35±4 at +3.0°C. In contrast, interclutch interval decreased from 17±2 days at

+0.0°C to 14±1 days at +1.5°C in the high CO_2 group before increasing to 19±6 days at +3.0°C.

On average pairs in the control temperature (+0.0°C) produced 1017±26 eggs per clutch (Fig. 3b). There was a trend for the number of eggs produced per clutch to decrease with increasing temperature (Fig. 3b). The decline was most obvious in the high CO_2 group, which decreased from 872 eggs per clutch at +0.0°C to just 12 eggs in the only clutch that was produced at 3.0°C. Despite the large decrease in the number of eggs produced, no treatment groups were significantly different from control +0.0°C (Table 3). Across all treatments, female weight had a significant positive effect on the number of eggs produced per clutch (Table 3).

Fertilization success was generally high, with fertilization being above 95% for 8 out of the 9 treatment groups. Moderate +3.0°C was the only group to exhibit a significant difference from control +0.0°C (Table 3) with fertilization being 51.42±48%.

Egg area was affected by a significant interaction between temperature and CO_2 (Table 3, Fig. 3c). At +0.0°C egg area increased with CO_2, though not linearly, with moderate +0.0°C producing the largest eggs overall and high +0.0°C producing an egg area intermediate to the moderate and control CO_2 treatments. Egg area declined with increasing temperature in all CO_2 treatments (Table 3, Fig. 3c). The moderate group exhibited the greatest reduction in egg area with increasing temperature with egg area of the +1.5°C and +3.0°C groups 83% and 75% of the moderate +0.0°C eggs. The control and high CO_2 displayed a similar trend with egg area of the control +1.5°C and +3.0°C groups being 92 and 74% and high groups being 88% and 72% of their +0.0°C groups respectively (Fig. 3c). Reproductive output showed a similar pattern to the number of eggs produced, with temperature having a negative effect on output, however only the control +3.0°C was different from the control +0.0°C (Table 3, Fig. 3d). There was no effect of CO_2 on reproductive output (Table 3).

More than 85% of the clutches produced in +0.0°C survived to hatching regardless of the CO_2 treatment. However, the number of clutches that survived to hatching markedly declined with increasing temperature (Table 4, Fig. 3e). No clutches survived to hatching at moderate or high +3.0°C and only one clutch survived to hatching in control +3.0°C (Fig. 3e). Egg survival to hatching was quite low, the highest average survival being 47% in the control CO_2 +0.0°C. Egg survival decreased with increasing CO_2 down to 29% survival in high +0.0°C (Table 4, Fig. 3f). In addition, temperature increase also decreased egg survival to 7% in the control and high CO_2 +1.5°C and no survival in the moderate CO_2 +1.5°C or the +3.0°C groups (Table 4, Fig. 3f). There was insufficient reproduction in the moderate 1.5°C and the +3.0°C treatment groups for significant differences to be detected.

Offspring characteristics

No clutches survived to hatchling in moderate CO_2 +1.5°C or the +3.0°C groups, consequently only the +0.0°C and control and high +1.5°C were analyzed. Both temperature and CO_2 had a significant effect on hatchling length (Table 5, Fig. 4a). Newly hatched larvae in the high +0.0°C and in the control +1.5°C groups were significantly shorter than the control +0.0°C (Fig. 4a). Elevated CO_2 but not temperature, had a significant negative impact on yolk area, with both the moderate and high +0.0°C treatment group larvae having smaller yolk reserves compared to control +0.0°C (Table 5, Fig. 4b).

Adult body and reproductive condition

Neither Fulton's K body condition factor or hepatosomatic index (HSI) were significantly affected by either temperature or CO_2, for reproductive females (Table 6, Fig. 5a,b). Fulton's K and HSI levels were generally high and only reduced at the most extreme treatment, high CO_2 +3.0°C. Temperature significantly effected gonadosomatic index (GSI) and there was an interaction between temperature and CO_2 treatment (Table 6). At +0.0°C, moderate CO_2 was significantly different from all other treatment groups (Fig. 5c). GSI was unaffected by temperature in control CO_2. At moderate CO_2, GSI was highest at +0.0°C and declined at the higher temperature. In contrast, GSI was highest at +1.5°C before decreasing at +3.0°C in the high CO_2 treatment (Fig. 5c).

Plasma E_2 concentrations

Plasma E_2 concentrations were significantly negatively affected by increasing temperature (Table 6). Concentrations in the +3.0°C were significantly lower than the +0.0°C E_2 concentrations (Fig. 5d). Factor analysis identified two main trends in the variance of plasma E_2 concentrations and gonadal development. First, that there was a negative relationship between the presence of stage 2 oocytes and a positive relationship between the presence of stage 4 and 5 oocytes with plasma E_2 concentration (Table 7) accounting for nearly 50% of the variation. The second factor, accounting for 25% of the variation, was influenced by the presence of stage 3 oocytes (Table 7). Multiple regression analysis showed significant relationship between factor 1 and plasma E_2 concentration ($F_{2,47}$=25.63, p<0.0001) which included a significant relationship to factor 1 ($F_{1,47}$=7.11, p<0.000001) but not factor 2 ($F_{1,47}$=-0.88, p=0.38).

Discussion

Higher temperatures and elevated CO_2 can act additively or synergistically to reduce individual performance (Pörtner & Farrell, 2008). Previous studies have found that reproduction declines at elevated temperatures in a range of marine fishes (Donelson *et al.,* 2010; Hilder & Pankhurst 2003; Van Der Kraak & Pankhurst 1997). Similarly, increased CO_2 is predicted to increase the energy required for maintaining homeostasis, and therefore reduce the amount of energy available for reproduction (Ishimatsu *et al*., 2008, Melzner *et al*., 2009; Pörtner, 2012). Despite this prediction, recent studies have found that exposure to increased CO_2 does not, on its own, cause reproduction to decline in fish (Sundin *et al.,* 2012; Frommel *et al.,* 2010; Miller *et al.*, 2013; Schade *et al.,* 2014). This study is the first to examine the interaction between temperature and CO_2 on reproduction in reef fish for an entire reproductive season. We found that the interaction between CO_2 and temperature was complex, but that overall, elevated temperature had a much greater effect on reproduction than did projected future CO_2 levels (Table 8). At control temperatures there was an apparent decline in reproductive output and offspring quality with increasing CO_2. At +1.5°C above current-day temperatures, breeding pairs in the moderate CO_2 treatment didn't produce successful clutches; however the control and high CO_2 pairs at this temperature continued to reproduce, though at a reduced rate compared to the same CO_2 treatments at control temperatures. By far the most obvious result from this study, was the complete cessation of reproduction at +3.0°C above current-day summer average temperature, irrespective of CO_2 level.

Reproductive and offspring characteristics: CO_2

In a previous experiment, Miller *et al.* (2013) observed an increase in reproduction at elevated CO_2 levels similar to those used in this experiment. A similar increase in reproduction has since been documented in the Three-spine stickleback (Schade *et al.,* 2014) and elevated CO_2 resulted in increased levels of reproductive hormones in Atlantic cod (Preus-Olsen *et al.,* 2014). Together, these results suggest that stimulation of reproduction by elevated CO_2 could occur in a variety of fish species from a range of families.

In this study, however, reproduction in the high CO_2 group was not significantly increased compared to the control or moderate groups (at control temperatures matching Miller *et al*. 2013). In this instance, the control and moderate groups doubled their reproductive activity compared to Miller *et al.* (2013), while the high group maintained reproductive levels in comparison to the previous study. The high CO_2 group did produce a similar number of eggs per clutch, and more clutches over the season, compared to Miller *et al.* (2013). The reason for the increase in reproductive performance of the control and moderate CO_2 groups compared to reproductive performance of control and moderate pairs in Miller *et al*. (2013) is unclear, but may be related to difference in the time required to acclimate to laboratory conditions. Breeding of wild-caught fish can improve with time in captivity, which could explain why the control and moderate group performed better in their second year of captivity (this study) compared with the earlier study (Miller *et al*., 2013). Behavioural studies show that reef fish exposed to CO_2 >700µatm tend to be bolder and more active (reviewed Munday *et al*., 2012) which may compensate for the stress response to captivity (Pankhurst & Van Der Kraak, 1997), leading to greater breeding in the first year in this group. Whatever the mechanism, our results suggest that examining just one reproductive season may not provide a full picture of the effect of elevated CO_2 on reproduction.

Unlike our previous study (Miller *et al.,* 2013) we also detected significant negative impacts on reproduction, with clear declines in egg survival and yolk provisioning. A similar decline in embryonic survival has recently been seen in a temperate goby (Forsgren *et al.*, 2013), but was not detected in a closely related species, *Amphiprion percula*, (Munday *et al.*, 2009b). The decline detected here equates to the high CO_2 +0.0°C group having less than half the number of surviving eggs per clutch (~250 eggs) compared to the control group (~540 eggs). A decline in reproductive output of this magnitude, if it occurs in wild populations, could potentially have a significant effect on population replenishment.

Further to the decline in embryonic survival, the larvae that were produced under high CO_2 were both shorter and had less yolk compared to the control and moderate CO_2 larvae. A reduction in yolk area was also detected in *A. percula* larvae reared at similar CO_2 levels (Munday *et al.,* 2009b). Yolk reserves provide the energy for growth until the larvae are able to feed, therefore a reduction in yolk provisioning could lead to reduced somatic growth at least in the early larval stage. Yolk reserve is also a good indicator for future growth and performance (Hoey & McCormick, 2004; Grorud-Colvert & Sponaugle, 2006). In addition, the high CO_2 offspring were significantly shorter at hatching compared to control offspring. Hatchling length is a key fitness-related trait (Miller *et al.*, 1998). Reductions in both yolk reserve and hatchling length could reduce juvenile performance, potentially increasing mortality.

Reproductive characteristics: Temperature and interaction

The most obvious result from our data was the negative impact of increasing temperature on every reproductive characteristic investigated, except fertilization success, regardless of CO_2 level. This was particularly obvious in the decline in number of eggs produced per clutch, with an increase of +3.0°C reducing the egg output of the control group by 75%. Even more startling was the decline in the number of eggs that survived to hatching. An increase of +1.5°C reduced survival to hatching from ~49% to ~7% in the control group. The same temperature increase resulted in no surviving eggs in the moderate CO_2 group, and at +3.0°C there were no surviving eggs regardless of CO_2 treatment. This trend of declining reproduction with increasing temperature has been shown in a number of tropical and temperate fishes (Donelson *et al*., 2010; Lansteiner & Kletzl, 2012; Warren *et al.,* 2012) and in other ectothermic animals (Snell 1986; Lee *et al.,* 2003). Given this trend, there could be serious declines in fish populations by 2100, through reduced reproduction, unless there is sufficient scope for thermal acclimation or adaptation of reproduction over the next few decades.

No studies have yet examined the potential for genetic adaptation of reproduction in fishes to ocean warming. However, one study has tested the potential for acclimation of reproduction to projected future warming in a reef fish. Donelson *et al.* (2014) found that reproductive traits in *Acanthochromis polyacanthus* were restored to control levels when fish complete development and are reaed their entire life at +1.5°C, but there was no reproductive acclimation when fish were reared at +3.0°C for their entire lives. Consequently, there appear to be constraints on the potential for acclimation, at least for some reef fishes, particularly at the higher temperatures (+3.0°C) that caused the greatest declines in reproduction in our study. Whether there is potential for transgenerational acclimation of reproduction is currently unknown.

As with elevated CO_2, we detected a significant negative effect of increased temperature on hatchling length in the control CO_2 group, and a similar, though non-significant, trend in the high CO_2 group. There may be a minimum size for hatchling length, similar to the minimum or optimal length required for metamorphosis of juveniles in fish and other species (Chambers & Leggett, 1987; Altwegg & Reyer, 2007). If so, the effect of elevated CO_2

treatment may have already reduced hatchling length close to the minimum viable length, to a point that increased temperature did not have a further significant impact. This hypothesis is supported by the declines in yolk provisioning that occurred in +1.5°C control offspring, not being present in the +1.5°C high CO_2 offspring. A minimum energy requirement may be needed for embryo's to survive to hatching.

Potentially the most surprising result in this study was the cessation of reproduction in the moderate CO_2 +1.5°C group. This was not due a delay in reproduction, as there was reproduction in this group prior to experimental temperatures being attained. The fact that the moderate CO_2 +0.0°C and the control +1.5°C groups both reproduced suggests that, on their own, neither stressor is enough to restrict reproduction. However, when the two occur in combination they cause sufficient stress on the organism, causing reproduction to cease. Interestingly, despite the clear decline in reproduction in the moderate CO_2 +1.5°C, there was not a significant decline in plasma E_2 concentrations in this group. This suggests that changes in concentrations of this particular sex steroid are not responsible for the decline in reproduction at moderate CO_2 +1.5°C. One possible explanation for the cessation of reproduction in this group, but not the high CO_2 +1.5°C, is that the moderate CO_2 level lies below the threshold at which physiological acclimation occurs. In Miller *et al.* (2013) we suggested that the increased reproduction in the high CO_2 group could be a hormetic response. It is possible that at the high CO_2 level, a change, caused by the increased CO_2 occurs that "switches on" reproduction. This switch could explain why the high CO_2, but not the moderate CO_2 group reproduced at +1.5°C.

There is a CO_2 threshold at which the behavioural impacts of elevated CO_2 begin to occur, somewhere between 600 and 700µatm in most reef fishes studied to date (Munday *et al.,* 2010). It is possible that whatever causes the change in behaviour (hypothesized to be disrupted neural activity, Nilsson *et al.,* 2012; Hamilton *et al.,* 2014) could also cause the stimulation of reproduction. At a more practical level, the cessation of reproduction at projected mid-century CO_2 and temperature levels is quite disturbing. The synergistic effects of these stressors could result in reproductive failures for tropical fishes within the next 40 years potentially impacting on commercial and non-commercial species alike. While there is evidence for transgenerational acclimation of life history-traits to ocean acidification (Miller *et al.*, 2012), and the at least potential for reproductive acclimation to moderate warming (+1.5°C)(Donelson *et al*., 2014), as yet, there is no evidence for acclimation to both these stressors in combination.

Hormonal and physiological impacts

The results suggest that reproductive, but not physiological condition, of females caused the changes in reproductive and offspring characteristics. First, elevated temperature resulted ina decrease in E_2 concentrations. Moreover, there was a strong correlation between E_2 concentration and the average number of clutches produced per pair. In contrast, there was little effect of elevated CO_2 or temperature on the physiological condition of either reproductive or non-reproductive females. Both Fulton's K body condition index and hepatosomatic index were high for all treatment groups, indicating no decline in body condition or energy stores. This suggests that the dramatic declines in reproductive outputobserved at higher temperature were not due to energetic constraints in the females. Previous studies have shown that under elevated temperatures the enzyme CYP19 aromatase, which catalyses the irreversible conversion of testosterone into E_2 in inhibited (Watts *et al.,* 2004). Inhibition of CYP19 aromatase will result in a decline in E_2 synthesis, and a subsequent reduction in vitellogenesis and oocyte maturation (Piferrer & Blázquez, 2005;

Yaron & Levavi-Sivan, 2011). Hence the results strongly suggest that a reduction of plasma E_2 concetrations, likely through inhibition of CYP19 aromatase may be the cause for decrease reproduction at higher temperature in *A. melanopus*.

Conclusions

This is the first time that the potential interactive effects of projected future CO_2 and temperature conditions have been tested in regards to fish reproduction. As with similar studies conducted on invertebrate reproduction (Chua *et al.*, 2013; Byrne *et al*., 2009), we found that temperature had a much stronger impact that CO_2. Our data, similar to other studies on tropical fish, showed that there was complete reproductive failure at +3.0°C above the current-day average temperature. Given that sea surface temperatures within the tropics are projected to rise up to +3.0°C (Ganachaud *et al.,* 2011) by 2100, there could be significant consequences for reproduction in tropical fish populations. Nevertheless, we did detect interactions between temperature and CO_2 at the combined moderate levels. Previous studies have shown that when temperature has increased +1.5°C that reproduction is reduced, as we saw in the control CO_2 group. Yet when the extra stress of increased CO_2 was added, and without any compensatory mechanisms, there appears to be a major reproductive failure. Our data suggest that, without reproductive acclimation or adaptation, there could be reproductive failure for this species as early as the middle of the century. These results reinforce the importance of examining how multiple stressors will interact, so that accurate climate change predictions can be made.

Our results also show that, for this species, there is no direct correlation between physiological condition and reproductive response and that one will not necessarily predict the other in future warmer and more acid conditions. Reproduction involves a complex series of interactions between environmental conditions and hormonal pathways. Further research will be required to determine the mechanisms responsible for declining reproduction at higher temperature, but it will likely involve thermal sensitivity of hormonal pathways. As yet there is no easy way to predict how reproduction will respond to climate change scenarios other than to experimentally test the population.

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Table 1: Seawater parameters for adult *Amphiprion melanopus* held under control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments. Temperature, salinity, total alkalinity and pH_{NBS} were measured in situ, while pCO_2 was calculated using CO2SYS.

Treatment	Temp (°C)	Salinity (ppt)	Total alkalinity (µmol kg^{-1} SW)	pH_{NBS}	pCO_2 (µatm)
Control +0.0°C	28.4±0.01	33.32±0.12	2058±16	8.15±0.005	400±6
Control +1.5°C	29.8±0.02	33.32±0.12	2064±16	8.14±0.005	411±6
Control +3.0°C	31.4±0.02	33.32±0.12	2077±16	8.12±0.005	441±7
Moderate +0.0°C	28.5±0.01	32.7±0.12	2152±10	8.00±0.007	634±13
Moderate +1.5°C	30.1±0.01	32.7±0.12	2117±7	8.00±0.006	642±12
Moderate +3.0°C	31.5±0.01	32.7±0.12	2130±8	8.00±0.007	658±13
High +0.0°C	28.5±0.01	33.62±0.09	2168±7	7.81±0.008	1087±25
High +1.5°C	29.8±0.02	33.62±0.09	2167±7	7.79±0.008	1126±24
High +3.0°C	31.5±0.01	33.62±0.09	2169±7	7.78±0.008	1191±27

Table 2. ANCOVA (type III) results for the average number of clutches produced by each adult pair kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments.

Treatment	DF	Sums of Squares	Mean Squares	F-value	p-value
Weight	1	1.252	1.252	0.095	0.7619
Temperature	2	207.932	103.9663	7.856	<0.01*
CO_2:Temperature	4	16.702	5.176	0.316	0.8629
Residuals	18	238.1976	13.233		

Table 3. Linear mixed effects model tables for the reproductive characteristics from adults kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments. Treatment groups that are significantly different to Control +0.0°C are marked by *.

Characteristic	Variable	Value	SE	DF	t-value	p-value
Interclutch Interval	(Intercept)	17.811	0.745	161	23.921	<0.0001*
	Control +1.5°C	1.174	1.669	19	0.703	0.4905
	Control +3.0°C	12.722	0.980	19	12.982	<0.0001*
	Moderate +0.0°C	-3.997	0.897	19	-4.458	<0.001*
	Moderate +1.5°C	4.678	3.832	19	1.221	0.2371
	Moderate +3.0°C	69.963	80.941	19	0.864	0.3982
	High +0.0°C	-6.249	0.816	19	-7.662	<0.0001*
	High +1.5°C	-0.319	1.763	19	-0.181	0.8582
	High +3.0°C	-5.784	5.416	19	-1.068	0.2989
Number of Eggs	(Intercept)	369.260	353.156	157	1.046	0.2974
	Female Weight	24.495	9.979	17	2.455	<0.05*
	Control +1.5°C	-230.128	196.044	17	-1.174	0.2566
	Control +3.0°C	-486.441	313.976	17	-1.549	0.1397
	Moderate +0.0°C	74.833	193.248	17	0.387	0.7034
	Moderate +1.5°C	95.137	388.244	17	0.245	0.8094
	Moderate +3.0°C	-322.907	409.861	17	-0.788	0.4416
	High +0.0°C	-139.385	214.176	17	-0.651	0.5239
	High +1.5°C	172.361	258.544	17	0.667	0.5139
	High +3.0°C	-209.533	450.492	17	-0.465	0.6477

Fertilisation success	(Intercept)	90.160	3.791	157	23.783	<0.0001*
	Control +1.5°C	9.409	6.405	18	1.469	0.1591
	Control +3.0°C	7.322	8.223	18	0.890	0.3850
	Moderate +0.0°C	9.362	6.161	18	1.520	0.1460
	Moderate +1.5°C	-8.931	13.081	18	-0.682	0.5035
	Moderate +3.0°C	-55.415	12.019	18	-4.611	<0.001*
	High +0.0°C	4.527	3.791	18	1.194	0.2479
	High +1.5°C	-3.439	8.528	18	-0.403	0.6915
	High +3.0°C	-2.009	13.196	18	-0.152	0.8807
Egg Area	(Intercept)	2.563	0.061	1627	41.684	<0.0001*
	Female Weight	-0.014	0.002	174	-7.943	<0.0001*
	Control +1.5°C	-0.277	0.034	174	-8.194	<0.0001*
	Control +3.0°C	-0.794	0.076	174	-10.428	<0.0001*
	Moderate +0.0°C	-0.095	0.032	174	-3.011	<0.01*
	Moderate +1.5°C	-0.197	0.075	174	-2.621	<0.01*
	Moderate +3.0°C	0.338	0.117	174	2.897	<0.01*
	High +0.0°C	-0.128	0.036	174	-3.571	<0.001*
	High +1.5°C	0.018	0.043	174	0.413	0.6804
	High +3.0°C	0.184	0.157	174	1.252	0.2122
Reproductive output	(Intercept)	1161.624	741.883	157	1.566	0.1194
	Female Weight	39.682	20.922	17	1.897	0.0750
	Control +1.5°C	-701.157	413.854	17	-1.694	0.1085
	Control +3.0°C	-1462.128	589.445	17	-2.481	<0.05*
	Moderate +0.0°C	104.453	411.792	17	0.254	0.8028
	Moderate +1.5°C	64.732	802.208	17	0.081	0.9366

Moderate +3.0°C	-398.727	793.082	17	-0.503	0.6216
High +0.0°C	-405.597	452.430	17	-0.896	0.3825
High +1.5°C	412.802	547.022	17	0.755	0.4608
High +3.0°C	-49.727	887.772	17	-0.056	0.9560

Table 4. Quasi-likelihood general linear model results for the average proportion of clutches that survived to hatching and average proportion of eggs that survived to hatching for adults kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments. Treatment averages that are significantly different from Control +0.0°C are marked with an asterix (*).

Treatment	Value	SE	DF	t-value	p value
Successful clutches (%)					
Control +0.0°C	-0.211	0.2	64	-1.125	0.2646
Control +1.5°C	-2.220	0.4	19	-5.217	<0.0001*
Control +3.0°C	-1.706	0.8	19	-2.069	0.0525
Moderate +0.0°C	0.020	0.3	19	0.079	0.9378
Moderate +1.5°C	-24.661	116809.1	19	0.000	0.9998
Moderate +3.0°C	-24.54947	110522.5	19	0.000	0.9998
High +0.0°C	-0.444	0.3	19	-1.481	0.1549
High +1.5°C	0.348	0.6	19	0.555	0.585
High +3.0°C	-23.584	54075.0	19	0.000	0.999
Egg survival					
Control +0.0°C	-0.156	0.15	179	-1.050	0.2953
Control +1.5°C	-2.250	0.36	19	-6.230	<0.0001*
Control +3.0°C	-1.762	0.87	19	-2.018	0.0580
Moderate +0.0°C	-0.292	0.2	19	-1.451	0.1630
Moderate +1.5°C	-23.271	65787.90	19	0.000	0.9997
Moderate +3.0°C	-23.237	71691.82	19	0.000	0.9997
High +0.0°C	-0.659	0.23	19	-2.828	<0.05*
High +1.5°C	0.661	0.49	19	1.258	0.1904

High +3.0°C	-22.369	35076.46	19	0.000	0.995

Table 5. Linear mixed effects ANOVA results for physical characteristics (hatchling length and yolk area) of offspring resulting from adults kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C) or +1.5°C (30.0°C) water temperature treatments. No clutches survived to hatching in the Moderate +1.5°C or +3.0°C treatment groups. Significant effects ($p<0.05$) are denoted by *.

Characteristic	Variable	Value	Standard Error	DF	t-value	p-value
Hatchling length	(Intercept)	3.245	0.128	557	25.358	<0.0001*
	Female Weight	-0.000	0.004	64	-0.012	0.9901
	Control +1.5°C	-0.083	0.036	64	-2.322	<0.05*
	Moderate +0.0°C	0.047	0.057	64	0.820	0.4152
	High +0.0°C	-0.111	0.055	64	-2.004	<0.05*
Yolk area	(Intercept)	0.486	0.036	557	13.685	<0.0001*
	Female Weight	-0.002	0.001	64	-1.903	0.0615
	Control +1.5°C	-0.006	0.010	64	-0.623	0.5356
	Moderate +0.0°C	-0.032	0.016	64	-2.001	<0.05*
	High +0.0°C	-0.046	0.015	64	-3.078	<0.01*

Table 6. Fixed factor type III ANOVA table for adult physiological parameters. Adults were kept at Control, Moderate or High CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperatures. Significant effects ($p<0.05$) are denoted by *.

Characteristic	Variable	DF	MS	F-value	p-value
Fulton's K	CO_2	2	0.079	1.184	0.313
	Temperature	2	0.211	3.157	0.051
	CO_2:Temperature	4	0.024	0.356	0.839
	Residuals	54	0.067		
H.S.I.	CO_2	2	0.016	0.374	0.689
	Temperature	2	0.041	0.971	0.385
	CO_2:Temperature	4	0.038	0.887	0.478
	Residuals	54	0.042		
G.S.I	CO_2	2	3.365	2.266	0.114
	Temperature	2	11.655	7.848	<0.01*
	CO_2:Temperature	4	4.157	2.800	<0.05*
	Residuals	54	1.486		
17β-estradiol	CO_2	2	1765838	1.044	0.361
	Temperature	2	10229356	6.050	<0.01*
	CO_2:Temperature	4	1584219	0.937	0.452
	Residuals	43	1690826		

Table 7. Eigenvector values from factor analysis examining the trends in variance of gonadal development in relation to plasma 17β-estradiol concentration in female *A. melanopus*. The percentage of variation in plasma 17β-estradiol concentration explained by the first two factors in given. Gamete stages that contributed >70% to the factors are bolded.

	Factor 1	Factor 2
Gonadal cell stage	48%	25%
Stage 1 Oogonia	-0.691	0.499
Stage 2 Perinucleolus	**-0.793**	0.170
Stage 3 Cortical alveolous	0.007	**0.975**
Stage 4 Early vitellogenic	**0.891**	0.011
Stage 5 Late vitellogenic	0.719	0.204

Table 8. Summary table of the reproductive and physiological characteristics measured, the predicted response of the stressors (elevated temperature and CO_2) and the observed effect of the stressors. + symbols represent positive effects of the treatment, - symbol represent a negative effect of treatment, = symbol represents no difference between treatment and control.

	Predicted response		Observed response	
	Temperature	CO_2	Temperature	CO_2
Physical Characteristics				
Fulton's K	–	–	=	=
Hepatosomatic Index	–/+	–	=	=
Gonadosomatic Index	–	+	–	+
17β-estradiol concentration	–	+	–	+
Offspring				
Hatchling length	–	=	–	–
Yolk area	–	=	–	–
Reproductive characteristics				
Clutches produced	–	+	–	=
Interclutch interval	+	–	+	–

Eggs per clutch	−	+	=	=
Fertilization success	−	=	=	=
Egg area	−/+	=	−	−
Reproductive output	−/+	+	−	−
Successful clutches	−	=	=	=
Egg survival	−	=	=	=

Figure 1. The data collected at each stage of reproduction in *A. melanopus*, (a) reproductive adults (b) clutches produced, (c) eggs collected form reproductive adults and (d) the resulting hatchlings. Breeding pairs were kept at control, moderate or high CO_2 cross-factored with either +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) temperature treatments.

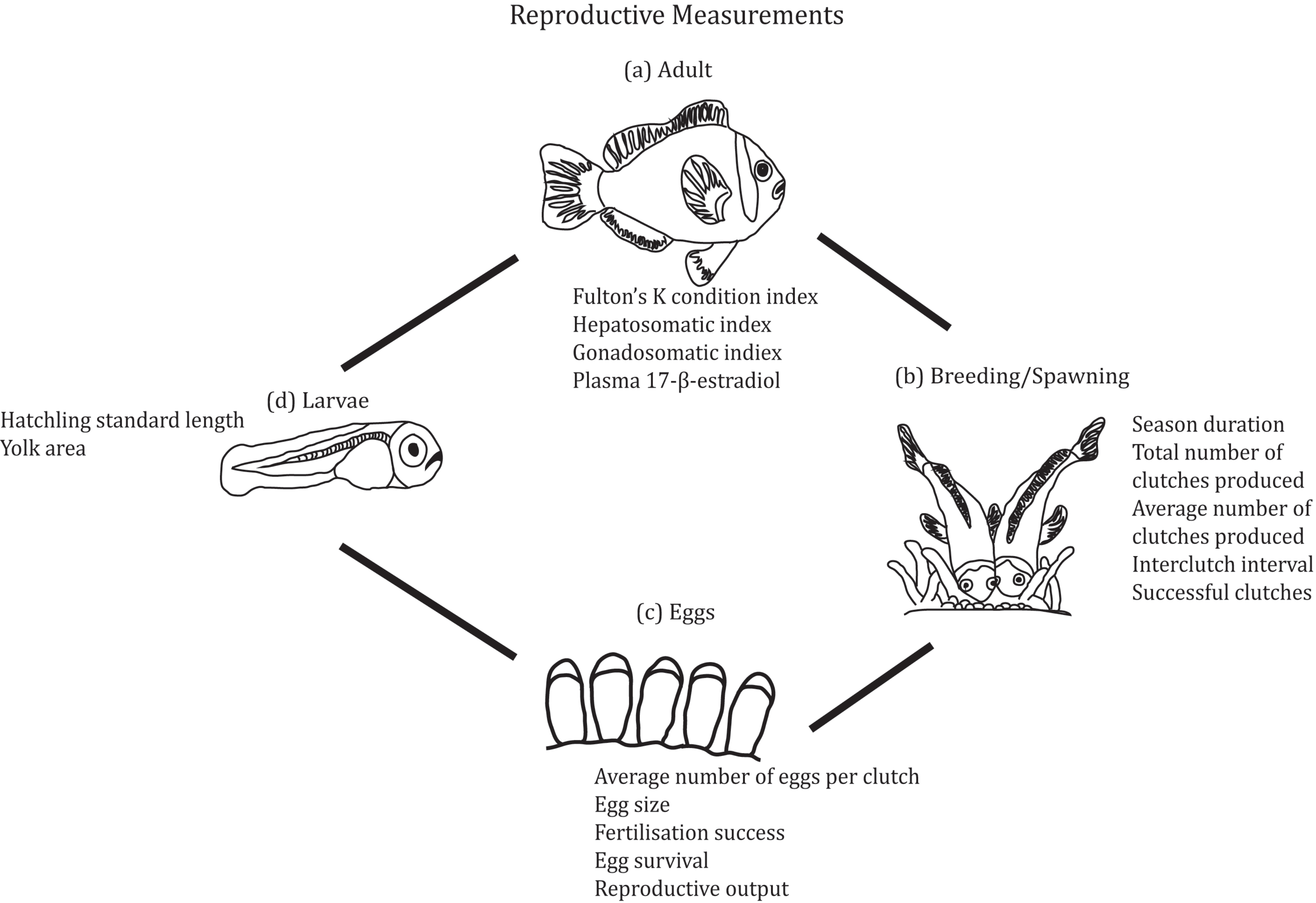
Figure 2. The total egg clutches produced per month across the breeding season for each CO_2 treatment at (a) +0.0°C (28.5°C), (b) +1.5°C (30.0°C) and (c) +3.0°C (31.5°C). Shown is the total number of clutches produced in each treatment for that month. The total number of clutches produced in each temperature by CO_2 treatment group and the number of pairs that reproduced in each treatment group are shown on the figure.

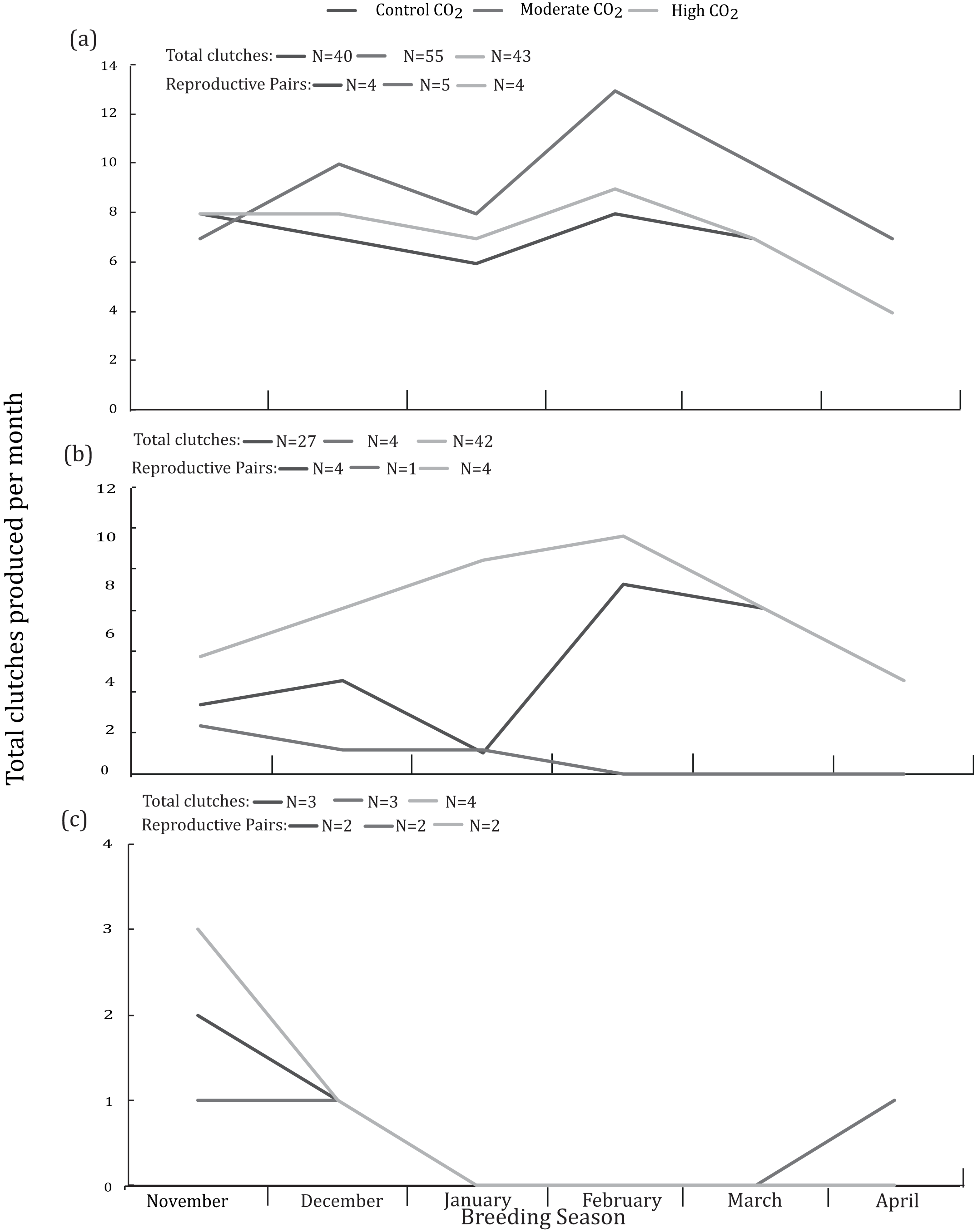
Figure 3. The reproductive characteristics of breeding pairs kept at control, moderate or high CO_2 cross factored with either +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) temperature treatments. The reproductive characteristics are: (a) the average number of clutches produced per pair for the breeding season, (b) the average number of eggs produced per clutch, (c) the average egg area (mm^2), (d) the average reproductive output, being the average egg area per clutch multiplied by the number of eggs in the clutch to give an estimate of energy (mm^2), (e) the average survival rate of the clutches produced and (f) the average egg survival. * denotes a treatment group that is significantly different from Control CO_2 +0.0°C.

Figure 4. Offspring characteristics from parents kept at control, moderate or high CO_2 cross-factored with either +0.0°C (28.5°C) or +1.5°C (30.0°C). No eggs survived to hatching in the moderate +1.5°C or the +3.0°C treatment groups, consequently they are not shown. The

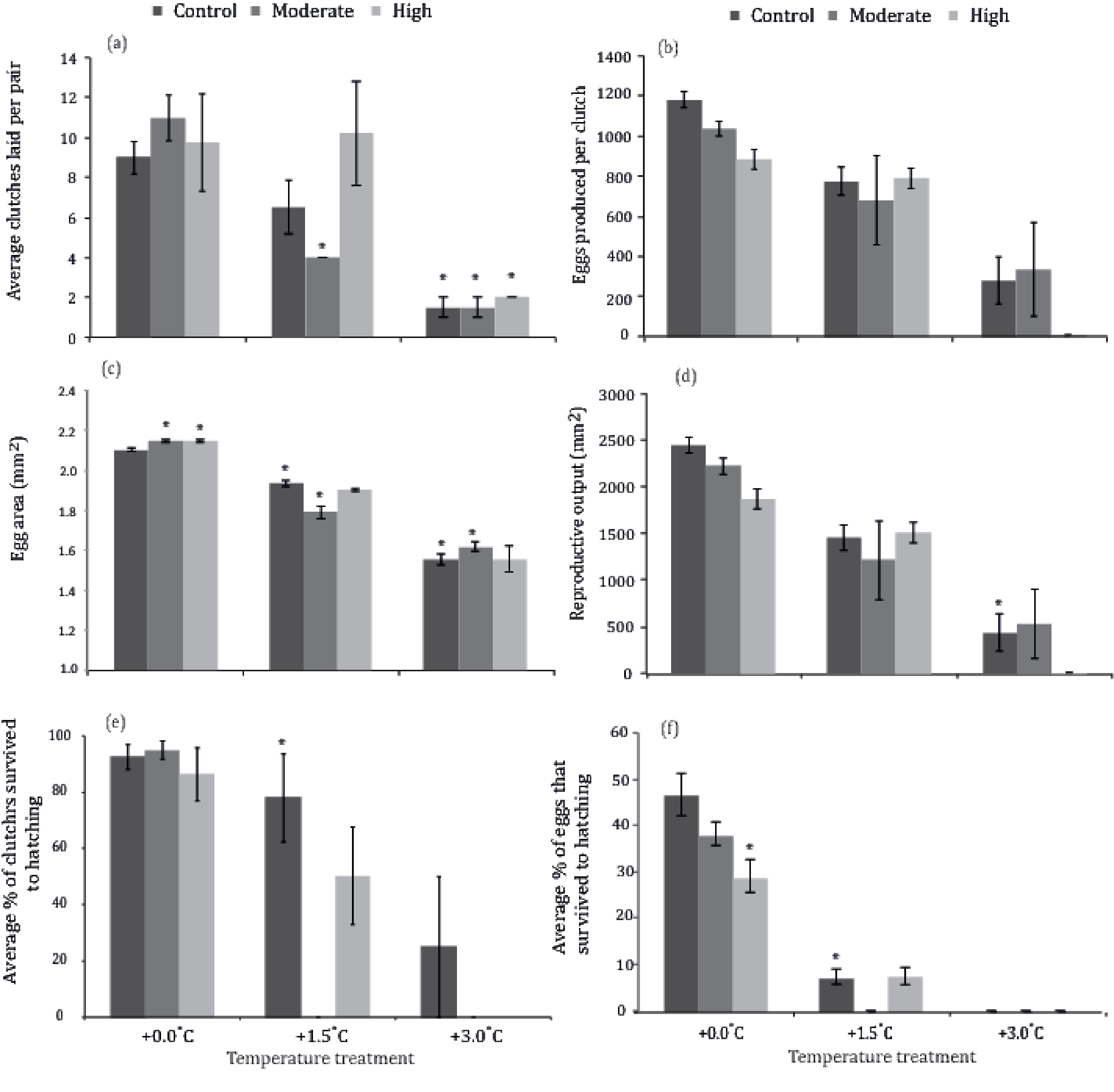
offspring characteristics were (a) hatchling standard length (mm) and (b) yolk area (mm^2). * denotes a treatment group that is significantly different from control CO_2 +0.0°C.

Figure 5. Adult physiological condition and hormone concentrations at the end of the breeding season, (a) Fulton's K body condition index, (b) hepatosomatic index, (c) gonadosomatic index and (d) plasma 17β-E_2 concentrations of females. * represent groups that are significantly different.

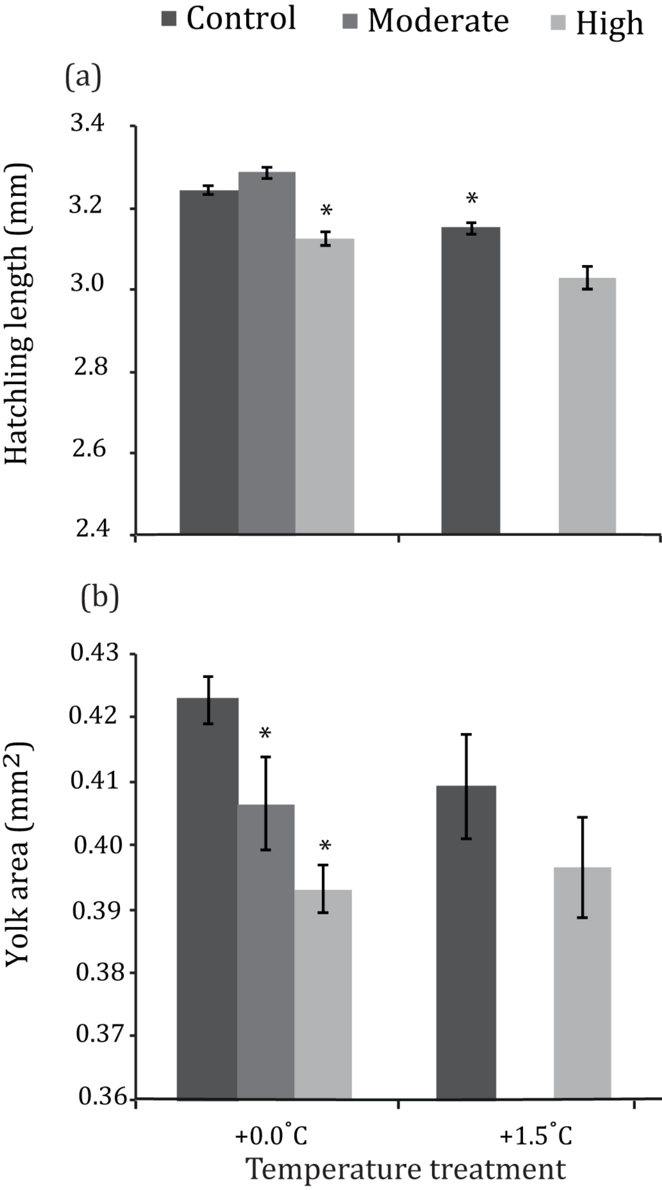

Reproductive Measurements
(a) Adult
Fulton’s K condition index
Hepatosomatic index
Gonadosomatic indiex
Plasma 17-β-estradiol
(b) Breeding/Spawning
Season duration
Total number of clutches produced
Average number of clutches produced
Interclutch interval
Successful clutches
(c) Eggs
Average number of eggs per clutch
Egg size
Fertilisation success
Egg survival
Reproductive output
(d) Larvae
Hatchling standard length
Yolk area



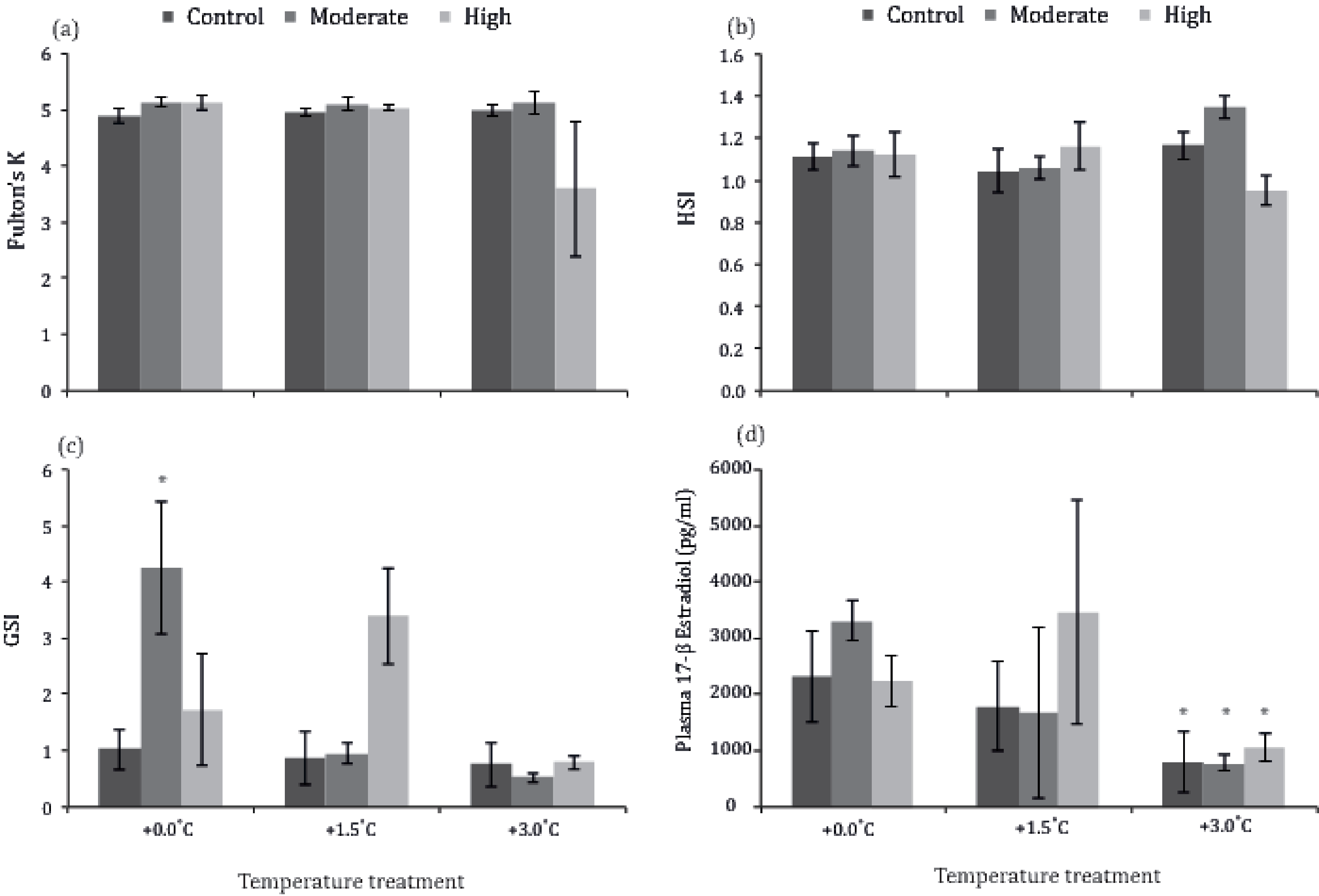
Control CO_2
Moderate CO_2
High CO_2
(a)
Total clutches: N=40 N=55 N=43
Reproductive Pairs: N=4 N=5 N=4
14
12
10
8
6
4
2
0
(b)
Total clutches: N=27 N=4 N=42
Reproductive Pairs: N=4 N=1 N=4
12
10
8
6
4
2
0
(c)
Total clutches: N=3 N=3 N=4
Reproductive Pairs: N=2 N=2 N=2
4
3
2
1
0
Total clutches produced per month
November
December
January
February
March
April
Breeding Season



Control
Moderate
High
(a)
Average clutches laid per pair
(b)
Eggs produced per clutch
(c)
Egg area (mm²)
(d)
Reproductive output (mm²)
(e)
Average % of clutchrs survived to hatching
(f)
Average % of eggs that survived to hatching
+0.0°C
+1.5°C
+3.0°C
Temperature treatment



Control
Moderate
High
(a)
Hatchling length (mm)
3.4
3.2
3.0
2.8
2.6
2.4
*
*
(b)
Yolk area (mm^2)
0.43
0.42
0.41
0.40
0.39
0.38
0.37
0.36
*
*
+0.0˚C
+1.5˚C
Temperature treatment



Control
Moderate
High
(a)
Fulton's K
(b)
HSI
(c)
GSI
*
+0.0°C
+1.5°C
+3.0°C
Temperature treatment
(d)
Plasma 17-β Estradiol (pg/ml)
* * *
Temperature treatment