Reproductive gene expression in a coral reef fish exposed to increasing temperature across generations

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Reproduction in marine fish is generally tightly linked with water temperature. Consequently, when adults are exposed to projected future ocean temperatures, reproductive output of many species declines precipitously. Recent research has shown that in the common reef fish, Acanthochromis polyacanthus, step-wise exposure to higher temperatures over two generations (parents: +1.5°C, offspring: +3.0°C) can improve reproductive output in the F2 generation compared to F2 fish that have experienced the same high temperatures over two generations (F1 parents: +3.0°C, F2 offspring: +3.0°C). To investigate how a step-wise increase in temperature between generations improved reproductive capacity, we tested the expression of well-known teleost reproductive genes in the brain and gonads of F2 fish using quantitative reverse transcription PCR and compared it among control (+0.0°C for two generations), developmental (+3.0°C in second generation only), step (+1.5°C in first generation and +3.0°C in second generation), and transgenerational (+3.0°C for two generations) treatments. We found that levels of gonadotropin receptor gene expression (Fshr and Lhcg) in the testes were reduced in developmental and transgenerational temperature treatments, but were similar to control levels in the step treatment. This suggests Fshr and Lhcg may be involved in regulating male reproductive capacity in A. polyacanthus. In addition, lower Fshb expression in the brain of females in all temperature treatments compared to control, suggests that Fshb expression, which is involved in vitellogenesis, is sensitive to high temperatures. Our results help elucidate key genes that facilitate successful reproduction in reef fishes when they experience a gradual increase in temperature across generations consistent with the trajectory of climate change.

Key words: Acanthochromis polyacanthus, climate change, gonadotropins, qRT-PCR, reproduction, transgenerational plasticity

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Introduction

Climate change is predicted to raise tropical sea surface temperatures by as much as 3°C by 2100 (Collins et al., 2013) with profound implications for the function and productivity of marine ecosystems (Harley et al., 2006; Pörtner et al., 2014). While many species will shift their geographic ranges as the oceans warm (Poloczanska et al., 2013), the populations that remain within the current range will experience elevated temperatures in the future. Adaptation to warmer
conditions could occur if a population has enough standing genetic variation (Munday et al., 2013), although there is concern that for many species the time required for genetic evolution may exceed the rate of ocean warming (Parmesan, 2006). Acclimation through phenotypic plasticity could be another important process that will assist organisms in coping with climate change (Huey et al., 2012; Munday et al., 2013; Crozier and Hutchings, 2014; Merila and Hendry, 2014). Beneficial acclimation occurs when physiological, morphological or behavioural phenotypes are plastically altered to better suit the environment (Angilletta, 2009). The phenotype of many animals can be adjusted in response to short-term changes in environmental conditions, such as daily or seasonal environmental fluctuations (reversible acclimation; Angilletta, 2009). However, environmental conditions experienced during early ontogeny can also induce phenotypic changes that persist throughout life (developmental plasticity) and parental exposure can alter the performance of their offspring in the same environment (transgenerational plasticity; Salinas et al., 2013; Torda et al., 2017).

Reproduction in fishes is tightly regulated by temperature, influencing processes such as gametogenesis, ovulation and spermatiation, embryogenesis and hatching, larval development, and sex determination (Van der Kraak and Pankhurst, 1997; Pankhurst and Munday, 2011). As fish are ectothermic and lack internal thermal regulation (Fry, 1967), changes in environmental temperature can have serious impacts on these critical reproductive processes (Davies et al., 1986; Van der Kraak and Pankhurst, 1997; Pankhurst and Munday, 2011; Zeh et al., 2012). Specifically, changes in environmental temperature are known to influence reproductive processes in numerous species of fish via the hypothalamo-pituitary-gonadal (HPG) axis. Following a temperature cue, gonadotropin-releasing hormones (GnRH) are synthesized in the hypothalamus and synthetically released onto gonadotropic cells in the pituitary, stimulating the release of pituitary gonadotropins: follicle stimulating hormone (FSH) and luteinising hormone (LH) (reviewed by Planas and Swanson, 2008; Levavi-Sivan et al., 2010; Zohar et al., 2010). In many fishes, dopamine (DA) has been shown to play an inhibitory role in releasing the gonadotropins, suggesting that FSH and LH release is dependent on the balance between DA and GnRH (reviewed by Dufour et al., 2010). FSH and LH stimulate gonadal function in both males and females by regulating the production of sex steroids (steroidogenesis) and gamete maturation (spermatogenesis and oogenesis, respectively). In male gonads, the enzyme Cyp11b1 converts the less active testosterone into 11-ketotestosterone (11-KT). In ovaries, the enzyme Cyp19a1a (aromatase) converts testosterone to 17β-estradiol (E2). Plasma levels of LH and FSH, in addition to the presence of their receptors in the gonads, vary depending on the sex, the level of sexual maturation of the fish, the phase of spermatogenesis or oogenesis, and the species (Planas and Swanson, 2008; Levavi-Sivan et al., 2010).

Due to the energetic cost and benefits of physiological optimization associated with reproduction, many species have evolved to reproduce within a narrow thermal range (Van der Kraak and Pankhurst, 1997; Browne and Wamigasekera, 2000; Visser et al., 2009). Although some species have already shifted their reproductive timing due to current changes in temperature (Parmesan and Yohe, 2003), other species may not have this ability and declines in quality and/or quantity of offspring, or reduced capacity for reproduction in general, are observed at temperatures outside the optimal thermal range (Giebelhausen and Lampert, 2001; Donelson et al., 2010; Miller et al., 2015). Consequently, warming associated with climate change poses a significant risk to population sustainability in these species. At the molecular level, higher than optimal reproductive temperatures can suppress expression of reproductive hormones and steroids (e.g. King et al., 2003; Pankhurst and King, 2010; Pankhurst and Munday, 2011). For example, when red seabream Pagus major, were exposed to elevated temperatures for up to 10 days, brain mRNA levels of GnRHI, and pituitary mRNA levels of GnRH-R, FshB and Lhb were reduced and there were lower serum levels of E2 (Okuzawa and Gen, 2013). Similarly, when reproductively active adult pejerrey Odontesthes bonariensis were exposed to elevated temperatures for 8 days, there were declines in transcript levels of Gnrh1 (brain) and FshB (pituitary) in both sexes, Lhb (pituitary) in males, and Fshr, Lhr and Cyp19a1a in female gonads, and reductions in plasma sex steroids (E2 and testosterone in females, 11-KT in males; Elsio et al., 2012). However, the effects of longer-term (i.e. developmental or transgenerational) exposure to high temperatures on transcript abundance of reproductive genes in the brain or gonads in these or other species have yet to be evaluated.

Recent studies have investigated the plasticity of physiological traits in marine fishes following developmental or transgenerational exposure to projected future warming (Donelson et al., 2011, 2012, 2016; Salinas and Munch, 2012; Shama and Wegner, 2014; Veilleux et al., 2015). However, few have assessed the potential for reproductive plasticity when exposed to elevated temperatures. Recently, Donelson et al. (2016) demonstrated that the coral reef damselfish, Acantthochromis polyacanthus, has the capacity for transgenerational reproductive plasticity when exposed to higher temperatures in a step-wise fashion over two generations, +1.5°C in the first generation and then +3.0°C in the second generation. In contrast, fish that were exposed to +3.0°C for two generations ceased to reproduce at all. Our study aimed to evaluate differences in gene expression between adult A. polyacanthus that possessed differences in reproductive capacity due to developmental and transgenerational exposure to elevated temperature. Importantly, we assessed gene expression of fish in the same step-wise transgenerational temperature treatment that was shown by Donelson et al. (2016) to possess partial acclimation of reproductive capacity. We predicted that the expression of reproductive genes in the brains and gonads would be downregulated in fish that were exposed to the same high temperature as their parents (i.e. two generations at +3.0°C) as no fish in this treatment were able to reproduce. By contrast, we predicted that expression of reproductive genes in the step-wise
treatment would be more similar to that of the controls, because they exhibited partial reproductive acclimation.

Material and methods

Study species and experimental design

Eight breeding pairs of *A. polyacanthus* (F0) were collected from the Palm Island region of the Great Barrier Reef, Australia, in July 2007. The Palm Island reefs are in the middle of the species range (18°37’S, 146°30’E) and have average yearly temperatures from 23.2°C to 28.5°C (Australian Institute of Marine Science temperature loggers 6–8 m; http://data.aims.gov.au). Breeding pairs were maintained in 60 L aquaria inside an environmentally controlled facility at James Cook University, Townsville, Australia.

The wild pairs produced offspring (F1) from December 2007 to February 2008. At 30 days post-hatching, clutches of F1 fish from each breeding pair were equally divided into one of three seasonally cycling temperature treatments: +0.0°C as well as ±1.5°C and ±3.0°C above average current seasonal temperatures (see Donelson et al. 2011 for more details). For 1 year after hatching, sibling fish were kept in groups of six in 40 L aquaria and then were reduced into pairs by the experimenter to reduce tank density. Mortality was very low among siblings, with >90% survival in all treatments. At 1.5 years post-hatching, fish were rearranged into non-sibling pairs from individuals from the same treatment, using an even number of individuals from each parental line. Fish reached maturity at 2 years old and reproduced during the austral summer 2011–2012, nesting sites in each temperature. Whole brains and gonads were homogenized and applied to PerfectPure Preclear columns (VWR, Murarrie, Australia). RNA was extracted according to manufacturer instructions, including an on-column DNase treatment. Total RNA quality and quantity was determined by absorbance readings on a NanoDrop Spectrophotometer (Invitrogen, Mulgrave, Australia) and an RNAse-free 1% agarose gel.

Total RNA for brains and gonads was normalized to a common concentration of 200 and 40 ng μl⁻¹, respectively. Complementary DNA (cDNA) was synthesized using 1 and 0.6 μg total brain and gonad RNA, respectively, and a blend of oligo(dT) and random primers in the iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Gladesville, Australia), as per manufacturer’s instruction. Each cDNA sample was 5-fold serially diluted twice in molecular grade water (Invitrogen, Mulgrave, Australia) to use as a working stock for quantitative reverse transcription PCR (qRT-PCR).

Aliquots of each original brain cDNA sample were combined and five 1:5 dilutions were performed to generate samples for a standard curve and for calculating PCR efficiency for each brain primer pair. This procedure was repeated separately for the gonad cDNA samples.

Intron-spanning primers for five reproductively genes in the brain (Fshb, Lhb, Gnrh1, Gnrhr and Ddc), and four reproductively genes in the gonads (Fsbr, Lhcr, Cyp19a1a and Cyp11b1) were designed using 3Prime (Koressaar and Remm, 2007; Untergasser et al., 2012) based on the genes from the assembled genome for *A. polyacanthus* (Schunter et al., 2016). In addition, intron-spanning reference gene primers were designed based on the most stably expressed genes in the *A. polyacanthus* transcriptome (see supplementary information Veilleux et al., 2015; Dvl1, Sin3b, Cnot1). Prior to the availability of the genome or transcriptome, primers for two reference genes (Ef1a and 18s rRNA) were designed using 3Prime (Koressaar and Remm, 2007; Untergasser et al., 2012) and were based on conserved regions of teleost genes obtained from the GenBank Public Database (Altschul et al., 1997). Primer sequences and details are listed in Table 1.

qRT-PCR was performed in triplicate 15 μl reactions using 1× SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Gladesville, Australia), 0.3 μM forward and reverse primers, and 10 ng cDNA. Using the Rotor-Gene Q (Qiagen Party Ltd, Chadstone, Australia) and a 100-well ring, the following qRT-PCR programme was used: 95°C for 30s, 50 cycles of 95°C for 5s and 58°C for 15s. Melting curve analysis was performed to test reaction specificity. Threshold Cq values and amplification efficiencies were calculated using LinRegPCR (version 2013.0; Ruijter et al., 2009). The qbasePLUS GeNorm software
Gladesville, Australia) with the following steps: pre-denaturation cDNA in a C1000 Thermal Cycler (Bio-Rad Laboratories, at 94 °C for 3 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 61°C for 30 s and extension at 72°C for 20 s; and, finally, a 10 min extension at 72°C. PCR products were visualized on a 1.5% agarose gel and sent to the Australian Genome Research Facility (AGRF) for purification and sequencing. The resulting trimmed 133 bp sequence was compared to those available in the GenBank Public Database (Alschul et al., 1997), with the highest match to Atlantic halibut, Hippoglossus hippoglossus, elongation factor 1 alpha, accession EU561358.1, e-value 8e-17.

Statistical analysis

The proportion of mature pairs that reproduced per treatment was compared using a chi squared test of homogeneity among treatments. Generalized least squares (GLS) ANOVA models were used to compare the expression of each gene in the brain and gonad. All samples were first analysed with sex (male or female) and treatment as fixed factors. Following this analysis, separate GLS models were run for male and female gene expression with treatment as a fixed factor. Due to experimental constraints and the nature of the A. polyacanthus breeding system, we could not be certain that fish were sampled at the same time within their reproductive cycles. Thus, the time since breeding for each fish was explored as a co-variate in the analysis, but no significant relationship was found. For all analyses, the gls function in the nlme package in R was used (version 3.4.1; Pinheiro et al., 2017).

Results

Reproductive success

The proportion of F2 adults that reproduced differed among treatments ($X^2 = 14.06, df = 3, P < 0.01$). Specifically, 4 of 10 (40%) control pairs, 1 of 9 (11%) developmental pairs, and 6 of 9 (67%) step treatment pairs reproduced, but 0 of 10 (0%) pairs reproduced in the transgenerational treatment. When breeding occurred, there was a tendency for pairs in warm treatments to produce fewer clutches over the season. Specifically, of the four control pairs that reproduced, two produced three clutches over the breeding season and the other two produced one. The single reproductive developmental pair produced two clutches and one of the reproductive step pairs produced two clutches while the other four produced only one.

Brain gene expression

The only gene assessed in the brain that exhibited a significant difference in expression depending on treatment was Fshb, when all samples were combined (Table 2) and when only females were considered (Table 2; Fig. 2C). Among the females, the significant treatment effect was due to developmental and step treatment fish having 0.8 (±0.3 SE) and 1.3 (±0.6 SE) fold lower expression compared to control, respectively (Fig. 2C). There was no significant effect of treatment in male brain gene expression (Table 2), however Ddc, Fshb and Gnrh1 showed decreased trends in expression in temperature treatments relative to control (Fig. 2B, D, J). In contrast, Gnrh had an increased trend in expression in the temperature treatments relative to control, with developmental and step expression both 1.2 (±0.3 SE) fold higher (Fig. 2H).
Gonad gene expression

*Cyp11b1*, *Cyp19a1a* and *Lhcgr* had significantly different expression between males and females (Table 2): *Cyp19a1a* expression was higher in females (+6.5 fold ± 0.5 SE; Fig. 3C and D) and *Cyp11b1* and *Lhcgr* had elevated expression in males (+5.4 fold ± 0.5 SE and +1.6 fold ± 0.4 SE, respectively; Fig. 3A, B and Fig. 3G, H). There were no significant treatment effects when evaluating all samples (Table 2). There were, however, significant differences among *Fshr* and *Lhcgr* expression in males (Table 2). Male *Fshr* expression in the control and step treatments were higher than developmental and transgenerational treatments (control: +0.7 fold ± 0.4 SE and +0.5 fold ± 0.3 SE, respectively; step: +0.7 fold ± 0.2 SE and +0.5 fold ± 0.1 SE, respectively; Fig. 3F). Similarly, male *Lhcgr* expression in the control and step treatments were higher than developmental and transgenerational treatments (control: +0.8 fold ± 0.3 SE and +1.3 fold ± 0.5 SE, respectively; step: +0.6 fold ± 0.2 SE and +1.2 fold ± 0.4 SE, respectively; Fig. 3H). Expression of both *Fshr* and *Lhcgr* in the males (Fig. 3F and H) showed similar trends to the proportion of pairs that were breeding, with control and step treatments elevated compared to developmental and transgenerational treatments.

There were no significant treatment effects among females (Table 2). Female *Lhcgr* tended to have an elevated trend in expression in step relative to developmental and transgenerational treatments (+1.4-fold ± 0.6 SE and +1.7-fold ± 0.9 SE higher, respectively; Fig. 3G); however, unlike male *Lhcgr*, control fish did not have an increased trend in expression compared to developmental and transgenerational treatments. Female *Cyp11b1* exhibited a trend toward elevated expression in the developmental treatment compared to all other treatments (+1.8-fold ± 0.6 SE, +1.8-fold ±0.9 SE, and

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**Table 1:** Quantitative reverse transcription PCR (qRT-PCR) brain and gonad target and reference genes, associated forward and reverse primer sequences and expected product length

| Type | Gene | Forward Primer (5’−3’) | Reverse Primer (5’−3’) | Expected product (bp) |
|------|------|------------------------|------------------------|-----------------------|
| Brain | Ddc  | Dopa Decarboxylase (an enzyme in the pathway that produces dopamine) | GTCCAGGCAACCACTCCAG | CTCCTAACAGAGAGCTGC | 110 |
|       | Fshb | Follicle Stimulating Hormone, Beta Polypeptide | CACCCAGGTCGGCCAGGC | ACCTCGAAGGACCAGTCAC | 105 |
|       | Gnri1/Sb-Gnri | Gonadotropin-Releasing Hormone 1 | CTGTCAGCAGCTCTGGATG | ACTGAAAGGCTGCTGCA | 115 |
|       | Gnhr | Gonadotropin-Releasing Hormone Receptor | TTCCTGCTCCACTGGCTAT | GAGCTCCATCCGGGTCTT | 148 |
|       | Lhb  | Luteinizing Hormone, Beta Polypeptide | AGAGCTGCTCTCGGAGAG | TACAGGTCCTGAGTGTC | 148 |
| Gonad | Cyp11b1 | Cytochrome P450, Family 11, Subfamily B, Polypeptide 1 (a.k.a. 11b-hydroxylase) | CAGCACTAGCAAGGAGTCTT | CAGAAATCCCTCGCACCCT | 137 |
|       | Cyp19a1a | Cytochrome P450, Family 19, Subfamily A, Polypeptide 1 (a.k.a arom/ aromatase) | CCGGACAGAGTCTCTTCTCA | CGAACTGGCTGAAGATACG | 86 |
|       | Fshr  | Follicle Stimulating Hormone Receptor | CCTCTCATACGCTCTCGGA | CGAGGGTGAAGGCGTACG | 95 |
|       | Lhcgr | Luteinizing Hormone/ Choriogonadotropin Receptor | TGAGCTGGCTAGAAACGGG | AGACTCGGACCTGGCTTC | 143 |
| Reference | Cnot1 | CCR4-NOT Transcription Complex, Subunit 1 | ATCCAAAACAGGAGCAGGCA | TCAGTGTCCAGTCCACAGCA | 95 |
|       | Dvl | Dishevelled Segment Polarity Protein 1 | AGTGAATCAGGCAAGGTCTCC | ACTGTGACTGGAGCATGG | 93 |
|       | Sin3b | SIN3 Transcription Regulator Family Member B | AACCAGGACAGCGCTCT | TGGATGTGGGCTAGCCT | 133 |
|       | 18s rRNA | 18s ribosomal RNA | TGGACAGGAGGCGACCA | AGAACGGCGCATAACGCC | 142 |
|       | Efla | Eukaryotic Translation Elongation Factor 1 Alpha 1 | ACGCTGGGCTTCAGAACA | TCCAGAATCGAGCAGC | 183 |
Table 2: Type III analysis of variance (ANOVA) testing differences in brain and gonad gene expression between sexes and/or treatments

| Tissue | Gene | Source | Df | ChiSq | Pr (Chisq) | Source | Df | ChiSq | Pr (Chisq) | Source | Df | ChiSq | Pr (Chisq) |
|--------|------|--------|----|-------|------------|--------|----|-------|------------|--------|----|-------|------------|
| Brain  | Ddc  | (Intercept) | 1 | 14.05 | <0.00      | (Intercept) | 1 | 10.69 | <0.00      | (Intercept) | 1 | 24.58 | <0.00      |
|        |      | Treatment | 3 | 0.94  | 0.82       | Treatment | 3 | 0.71  | 0.87       | Treatment | 3 | 2.84  | 0.42       |
|        |      | Sex       | 1 | 0.86  | 0.35       | Treatment: Sex | 3 | 0.42  | 0.94       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 5.82  | 0.12       |          |    |       |            |          |    |       |            |
|        | Fshb | (Intercept) | 1 | 52.77 | <0.00      | (Intercept) | 1 | 58.63 | <0.00      | (Intercept) | 1 | 35.20 | <0.00      |
|        |      | Treatment | 3 | 11.45 | 0.01       | Treatment | 3 | 12.72 | 0.01       | Treatment | 3 | 6.02  | 0.11       |
|        |      | Sex       | 1 | 0.04  | 0.85       | Treatment: Sex | 3 | 0.11  | 0.74       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 3.99  | 0.26       |          |    |       |            |          |    |       |            |
|        | Lhb  | (Intercept) | 1 | 5.26  | 0.02       | (Intercept) | 1 | 13.13 | <0.00      | (Intercept) | 1 | 3.52  | 0.06       |
|        |      | Treatment | 3 | 0.51  | 0.92       | Treatment | 3 | 1.26  | 0.74       | Treatment | 3 | 3.75  | 0.29       |
|        |      | Sex       | 1 | 0.11  | 0.74       | Treatment: Sex | 3 | 0.80  | 0.85       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 3.99  | 0.26       |          |    |       |            |          |    |       |            |
|        | Gnrh1| (Intercept) | 1 | 6.64  | 0.01       | (Intercept) | 1 | 4.20  | 0.04       | (Intercept) | 1 | 22.48 | <0.00      |
|        |      | Treatment | 3 | 0.71  | 0.87       | Treatment | 3 | 0.45  | 0.93       | Treatment | 3 | 3.68  | 0.30       |
|        |      | Sex       | 1 | 1.01  | 0.31       | Treatment: Sex | 3 | 0.80  | 0.85       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 3.99  | 0.26       |          |    |       |            |          |    |       |            |
|        | Gnrhr| (Intercept) | 1 | 21.66 | <0.00      | (Intercept) | 1 | 18.45 | <0.00      | (Intercept) | 1 | 8.51  | <0.00      |
|        |      | Treatment | 3 | 2.65  | 0.45       | Treatment | 3 | 2.26  | 0.52       | Treatment | 3 | 5.76  | 0.12       |
|        |      | Sex       | 1 | 0.73  | 0.39       | Treatment: Sex | 3 | 5.61  | 0.13       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 5.61  | 0.13       |          |    |       |            |          |    |       |            |
| Gonad  | Cyp11b1| (Intercept) | 1 | 6.92  | 0.01       | (Intercept) | 1 | 8.27  | <0.00      | (Intercept) | 1 | 124.90 | <0.00     |
|        |      | Treatment | 3 | 4.91  | 0.18       | Treatment | 3 | 5.87  | 0.12       | Treatment | 3 | 4.73  | 0.19       |
|        |      | Sex       | 1 | 60.87 | <0.00      | Treatment: Sex | 3 | 7.61  | 0.05       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 7.61  | 0.05       |          |    |       |            |          |    |       |            |
|        | Cyp19a1a| (Intercept) | 1 | 239.33| <0.00      | (Intercept) | 1 | 519.85| <0.00      | (Intercept) | 1 | 9.02  | <0.00      |
|        |      | Treatment | 3 | 0.46  | 0.93       | Treatment | 3 | 1.01  | 0.80       | Treatment | 3 | 1.65  | 0.65       |
|        |      | Sex       | 1 | 46.22 | <0.00      | Treatment: Sex | 3 | 2.30  | 0.51       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 2.30  | 0.51       |          |    |       |            |          |    |       |            |
|        | Fshr  | (Intercept) | 1 | 15.52 | <0.00      | (Intercept) | 1 | 9.13  | <0.00      | (Intercept) | 1 | 167.70 | <0.00     |
|        |      | Treatment | 3 | 0.96  | 0.81       | Treatment | 3 | 0.56  | 0.90       | Treatment | 3 | 9.46  | 0.02       |
|        |      | Sex       | 1 | 2.28  | 0.13       | Treatment: Sex | 3 | 0.48  | 0.92       |          |    |       |            |
|        |      | Treatment: Sex | 3 | 0.48  | 0.92       |          |    |       |            |          |    |       |            |
+1.2-fold ± 0.6 SE vs. control, step, and transgenerational treatments respectively; Fig. 3A).

**Discussion**

Maintaining reproductive performance at higher water temperatures will be critical for the persistence of marine species as the climate continues to warm. Although the reef fish *A. polyacanthus* can fully acclimate aerobic metabolism when both parents and offspring are exposed to the same elevated temperatures (Donelson et al., 2012; Veilleux et al., 2015), these fish were unable to reproduce at elevated temperatures; only when there was a more gradual increase in temperature over two generations did fish show improved reproductive capacity (Donelson et al., 2016). Our study aimed to understand how fish were able to adjust reproductive capacity across generations by evaluating the expression of five genes in the brain and four in the gonads of *A. polyacanthus* that are known to be associated with teleost reproduction. By elucidating the molecular mechanisms underpinning reproductive plasticity, we can better understand and predict which populations or species will be most at risk in the future. The step treatment, which experienced a temperature increase of 1.5°C in two successive generations, had a similar proportion of pairs that reproduced compared to control, whereas developmental and transgenerational treatments that were immediately exposed to a +3.0°C increase in temperature had fewer and no pairs reproducing, respectively. Although there were few differences in brain or gonad gene expression among treatments, some patterns emerged. When male and female expression was explored separately, among treatments, some patterns emerged. When male and female expression was explored separately, among treatments, some patterns emerged. When male and female expression was explored separately, among treatments, some patterns emerged. When male and female expression was explored separately, among treatments, some patterns emerged.

Instead, our results suggest that gonadotropin receptors in the male gonads may play a role in the ability to acclimate reproductive capacity and that brain *Fshb* expression could be a temperature-sensitive regulator of vitellogenesis.

Gonadotropins are critical for physiological action and exert their effects on gonads through their receptors, FSH-R and LH-R (Kumar and Trant, 2001). In adult male and female pejerrey, *O. bonariensis*, gonadotropin receptor gene expression decreased when exposed acutely to elevated water temperatures (+4°C and +8°C above the average peak reproductive temperature), though it was only significant for female *Fsbr* (Soria et al., 2008). Furthermore, the pejerrey did not spawn at elevated temperatures and had reduced plasma sex steroid levels, leading the authors to suggest that gonads are particularly sensitive to increased water temperatures. In our study, we also observed a decrease in gonadal gonadotropin receptor expression (*Lhcgr* and *Fshr*) in the two elevated temperature treatments that reproduced poorly, developmental and transgenerational, but not the more gradual step treatment, which showed intermediate reproductive capacity. Interestingly, this difference in *Lhcgr* and *Fshr* expression was only found within the male gonads. Donelson et al. (2010), found a significant reduction in the proportion of spermatozoa in testes of adult *A. polyacanthus* exposed to +3.0°C for 7 months. As gonadotropins stimulate gamete development and maturation, the elevated levels of receptor expression and superior ability to reproduce at +3.0°C in the step treatment compared to developmental and transgenerational treatments suggests that *Fsbr* and *Lhcgr* in the testes may play an important role in plastically altering the ability to reproduce at higher temperatures. Furthermore, *Lhcgr* and *Fsbr* in the testes had a similar trend in expression compared to the proportion of pairs that were able reproduce, suggesting that the reduced capacity and inability to reproduce when exposed developmentally or transgenerationally to +3.0°C, respectively, may be due primarily to limitations within the testes and not ovaries. Thus, we have identified testicular *Lhcgr* and *Fsbr* as potential biomarkers for reproductive plasticity in *A. polyacanthus*; however, to fully elucidate the role these genes and
Figure 2: Mean (±SE) log₂ brain gene expression (Ddc, Fshb, Lhb, Gnrhr and Gnrh1) for control, developmental, step and transgenerational Acanthochromis polyacanthus treatments. Note: some error bars are too small to be seen. Female samples are denoted with diamonds and males with squares. Gene expression relative to reference genes Cnot1 and Dvl1.
their encoded proteins play in reproductive capacity and their use as biomarkers, we recommend additional experiments in other species following incremental transgenerational exposure to high temperatures associated with climate change.

Gametogenesis is regulated by the gonadotropins FSH and LH, which are synthesized and released when GnRH stimulates cells of the pituitary gland (Planas and Swanson, 2008; Levavi-Sivan et al., 2010; Zohar et al., 2010). Furthermore, synthesis of the gonadotropins is thought to be dependent on the balance of the activating GnRH and inhibiting DA (Dufour et al., 2010). However, the precise function of FSH and LH in fish gametogenesis is not well understood, exhibiting differences in concentrations at various time points across

Figure 3: Mean (±SE) log2 gonad gene expression (Cyp11b1, Cyp19a1a, Fshr and Lhcgr) for control, developmental, step and transgenerational *Acanthochromis polyacanthus* treatments. Note: some error bars are too small to be seen. Female samples are denoted with diamonds and males with squares. Gene expression relative to reference genes Dvl1 and Ef1a.
both synchronous and asynchronous spawners (see Levavi-
Sivan et al., 2010 for review). We expected that Ddc (a gene
encoding an enzyme that converts L-DOPA into DA) and
Gnrh1 would show elevated and reduced expression in the
brain, respectively, in the two temperature treatments that
had fewer (developmental) and no (transgenerational) pairs
capable of reproducing compared to control. However, the
trends in brain expression across treatments for Ddc and
Gnrh1 were instead similar to each other and also to Fshb,
showing a general trend of reduced expression in the tem-
temperature treatments relative to control. Fshb was the only tested
genome in the brain in which there was a significant treatment
effect and it had reduced expression in treatments exposed to
+3.0°C among all individuals and among females. In rainbow
tout, FSH stimulated the incorporation of over twice as
much vitellogenin into ovaries compared to LH (Tyler et al.,
1991) and plays a primary role in mediating vitellogenetic
development (Tyler et al., 1997). In yellowtail kingfish treated with
recombinant FSH, females exhibited an increased trend in
ovocyte diameter compared to controls (Sanchís-Benlloch
et al., 2017). Donelson et al. (2016) found that when a subset
of the transgenerational A. polyacanthus were transferred to
control temperatures during reproduction, they produced sig-
ificantly smaller eggs compared to control. Thus, the reduction
in Fshb in female A. polyacanthus +3.0°C treatments suggests
that FSH, a regulator of vitellogenesis, is sensitive to increases
in temperature, regardless of whether experienced developmen-
tally, step-wise or transgenerationally.

The observed expression profile differences of Cyp11b1 and
Cyp19a1a between sexes is intuitive: Cyp11b1 is 5.4-fold sig-
nificantly greater in males as its encoded protein converts tes-
tosterone to the active metabolite 11-KT, while Cyp19a1a is
6.5-fold greater in females as its encoded protein converts tes-
tosterone to E2. There was no difference in the expression of
Cyp19a1a among treatments in females, despite reduced Fshb
expression in the higher temperature groups. Similarly, coho
salmon ovarian follicles incubated with FSH showed no differ-
ence in Cyp19a1a compared to control, but had significantly
lower E2 levels (Luckenbach et al., 2011). The authors sug-
pected that increases in E2, but not Cyp19a1a, could be due to
upregulation of other genes in the steroid biosynthesis cascade.
In thermally stressed Atlantic salmon, Salmo salar, there were
reductions in egg size, which was associated with reductions in
plasma E2 levels (King et al., 2003). Thus, despite no change in
Cyp19a1a among A. polyacanthus females, FSH may still cause
a reduction in E2 levels in the elevated temperature treatments
by affecting expression of other genes in the steroid biosyn-
thesis cascade, ultimately leading to reduced egg size.

Consistent with the results from Donelson et al. (2016),
here we show that A. polyacanthus can acclimate reproductive
capacity to +3.0°C if temperature is ramped in +1.5°C steps
across generations. This pattern of improved reproduction
matched the observation that male gonadotropin receptor
(Lhcgr and Fshr) gene expression in the gonads in the step
treatment were at control levels, and higher than the two other
elevated temperature treatments. This difference in Lhcgr and
Fshr gene expression in male rather than female gonads sug-
gests that spermatogenesis may be more thermosensitive than
oogenesis in A. polyacanthus. Furthermore, the expression pat-
tern of Lhcgr and Fshr indicate that plasticity of these genes
within the testes may improve reproductive capacity and further
research into the molecular mechanisms leading to improved
spermatogenesis following gradual transgenerational exposure
to increased temperatures should be explored. The fish used in
this study were sacrificed on the same date and thus not all
individuals were at the same reproductive stage. Therefore,
although Fshb, Fshr and Lhcgr showed significant differences
among treatments, the other genes assessed that were not sig-
nificant (Ddc, Lhb, Gnrh1, Gnrhr, Cyp11b1 and Cyp19a1a)
may still play a role in plasticity, but at more specific time
points during the reproductive cycle. Future studies could ex-
amine the expression of these genes throughout the reproduc-
tive cycle to test for a possible role in reproductive acclimation to
elevated temperatures. Here we identified the sex, tissue, and
genes that are likely involved in transgenerational plasticity of
reproductive capacity in A. polyacanthus, thus providing a
more targeted approach for assessing the effects of increased
temperature on this species and others, in both wild and labora-
tory settings. Furthermore, our findings highlight the need for
experimental approaches that increase temperature gradually,
or in several steps, to better understand how species will cope
with future climate change over relevant time scales.

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