Comparative Biochemistry and Physiology, Part A 272 (2022) 111280

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Accustomed to the heat: Temperature and thyroid hormone influences on oogenesis and gonadal steroidogenesis pathways vary among populations of Amargosa pupfish (*Cyprinodon nevadensis amargosae*)

Sean C. Lema [a,*], Michelle I. Chow [a], Andrew H. Dittman [b], Darran May [c], Madeline J. Housh [a]

[a] Biological Sciences Department, Center for Coastal Marine Sciences, California Polytechnic State University, San Luis Obispo, CA, USA
[b] Environmental and Fisheries Sciences Division, Northwest Fisheries Science Center, National Marine Fisheries Service, NOAA, Seattle, WA, USA
[c] Aquatic and Fishery Sciences, University of Washington, Seattle, WA, USA

ARTICLE INFO

Editor by Michael Hedrick

Keywords:
Temperature
Fish
Reproduction
Estrogen receptor
Evolution
Climate change

ABSTRACT

Many fish experience diminished reproductive performance under atypically high or prolonged elevations of temperature. Such high temperature inhibition of reproduction comes about in part from altered stimulation of gametogenesis by the hypothalamic-pituitary-gonadal (HPG) endocrine axis. Elevated temperatures have also been shown to affect thyroid hormone (TH) signaling, and altered TH status under high temperatures may impact gametogenesis via crosstalk with HPG axis pathways. Here, we examined effects of temperature and 3′-triiodo-$_L$-thyronine (T_3) on pathways for gonadal steroidogenesis and gametogenesis in Amargosa pupfish (*Cyprinodon nevadensis amargosae*) from two allopatric populations: 1) the Amargosa River – a highly variable temperature habitat, and 2) Tecopa Bore – an invariably warm groundwater-fed marsh. These populations were previously shown to differ in TH signaling profiles both in the wild and under common laboratory conditions. Sexually-mature pupfish from each population were maintained at 24 °C or 34 °C for 88 days, after which a subset of fish was treated with T_3 for 18–24 h. In both populations, mRNA abundances for follicle-stimulating hormone receptor and luteinizing hormone receptor were higher in the ovary and testis at 24 °C compared to 34 °C. Females from Tecopa Bore – but not from the Amargosa River – also had greater ovarian transcript abundances for steroidogenic enzymes cytochrome P450 aromatase, 3β-hydroxysteroid dehydrogenase, and 17β-hydroxysteroid dehydrogenase at 24 °C compared to 34 °C, as well as higher liver mRNA levels of vitellogenins and choriogenins at cooler temperature. Transcript abundances for estrogen receptors *esr1*, *esr2a,* and *esr2b* were reduced at 34 °C in Amargosa River females, but not in Tecopa Bore females. T_3 augmented gonadal gene transcript levels for steroid acute regulatory protein (StAR) transporter in both sexes and populations. T_3 also downregulated liver estrogen receptor mRNAs in females from the warmer Tecopa Bore habitat only, suggesting T_3 modulation of liver E_2 sensitivity as a possible mechanism whereby temperature-induced changes in TH status may contribute to shifts in thermal sensitivity for oogenesis.

1. Introduction

Actinoperygiian fishes often depend on temperature to regulate critical physiological processes including reproduction. Optimal temperatures for reproduction vary widely among fishes depending on the thermal dynamics of their habitat, latitude, and seasonality of reproduction (Pankhurst and Porter, 2003; Pankhurst and Munday, 2011). However, across fish species, reproduction is sensitive to temperature perturbations and exposure to above-normal temperatures has deleterious effects on reproductive function, even for fishes adapted to high temperature environments (Motani and Wainwright, 2015). Exposure of fish to unusually high or prolonged elevations in temperature can disrupt sexual differentiation (Kitano et al., 1999; Uchida et al., 2004; Huynh et al., 2019), alter seasonal maturation (Dorts et al., 2012; Webb et al., 1999), impair gametogenesis and ovulation, or inhibit spawning behaviors (Tveiten and Johnsen, 2001; Glasser et al., 2004; Suquet et al., 2005; Soria et al., 2008; Donelson et al., 2010; Alix et al., 2020). These impacts have been shown to be mediated by several interacting physiological mechanisms including metabolic energy allocation for reproduction (Pankhurst and Munday, 2011), activation of physiological

* Corresponding author.
E-mail address: slema@calpoly.edu (S.C. Lema).

https://doi.org/10.1016/j.cbpa.2022.111280
Received 17 May 2022; Received in revised form 20 July 2022; Accepted 21 July 2022
Available online 25 July 2022

stress responses via the hypothalamic-pituitary-interrenal (HPI) axis (Donelson et al., 2010; Pankhurst, 2011), and, critically, direct alterations to hypothalamic-pituitary-gonadal (HPG) axis hormone signaling (Van Der Kraak and Pankhurst, 1997; Pankhurst and Munday, 2011; Miranda et al., 2013; Alix et al., 2020; Servili et al., 2020; Zahangir et al., 2022).

Studies examining temperature effects on the HPG axis in fishes have documented significant decreases in plasma concentrations of 17β-estradiol (E_2) or testosterone (T) at high temperature (Tveiten and Johnsen, 2001; King et al., 2007; Soria et al., 2008; Vikingstad et al., 2016; Wylie et al., 2018; Hani et al., 2019; Bock et al., 2021; reviewed by Alix et al., 2020; Servili et al., 2020). Such declines in sex steroid concentrations may emerge both from shifts in the sensitivity of steroidogenesis pathways to gonadotropins or temperature-induced changes in steroidogenic enzyme activity. High temperature-induced reductions of gonadal luteinizing hormone (LH) and follicle-stimulating hormone (FSH) receptor expression have been observed in several fishes, suggesting reduced sensitivity of gonadal tissues to gonadotropins (Yamaguchi et al., 2007; Soria et al., 2008; Veilleux et al., 2018; Anderson et al., 2019; Bock et al., 2021). Supporting this idea, rainbow trout (*Oncorhynchus mykiss*) maintained at 18 °C were found to have reduced induction of steroidogenesis by a luteinizing hormone-releasing hormone (LHRH) analogue compared to fish at 12 °C (Pankhurst and Thomas, 1998). Similarly, female Arctic charr (*Salvelinus alpinus*) reared at 10 °C – a temperature that causes near complete inhibition of ovulation in this species – and injected with salmon gonadotropin-releasing hormone (sGnRHa) produced significantly less LH than those reared at 5 °C (Gillet and Breton, 2009). Such high temperature-associated changes in gonadotropin sensitivity may ultimately result in reductions in gonadal steroidogenesis. For instance, Atlantic salmon (*Salmo salar*) given a LHRH analogue had lower production of both testosterone (T) and 17α,20β-dihydroxy-4-pregnen-3-one, an oocyte maturation-inducing hormone in fish (e.g., Nagahama and Yamashita, 2008), when exposed to elevated temperature (King and Pankhurst, 2004).

Reduced gonadotropin stimulation of gonadal steroidogenesis, however, does not appear to be the sole endocrine mechanism mediating reproductive inhibition at elevated temperatures. Fish exposed to warmer temperatures can also have reduced gonadal expression of steroid acute regulatory protein (*star*) and P450 side chain cleavage enzyme (*cyp11a1*), as well as ovarian P450 aromatase (*cyp19a1a*) (Elisio et al., 2012; Anderson et al., 2012a, 2019; Mazzeo et al., 2014; Bock et al., 2021), which appears to result in lower circulating E_2 in females at elevated temperatures. Such lower E_2 subsequently leads to diminished vitellogenin egg yolk precursor and choriogenin egg envelope protein production by the liver for female fish exposed to higher temperatures (Pankhurst and King, 2010; Anderson et al., 2012b; Bock et al., 2021).

Less considered is that higher temperatures may also modulate other hormone pathways that interact with the HPG axis to lessen reproductive performance. The physiological actions of thyroid hormones (THs) in fish are sensitive to variation in temperature (e.g., Little et al., 2013; Lema et al., 2016; Zak and Manzon, 2019), and changes in TH iodothyroine deiodinase enzyme or TH receptor expression have been observed in fish experiencing warmer conditions (Johnston and Eales, 1995; Lema et al., 2016). In rainbow trout (*Oncorhynchus mykiss*), for example, fish maintained at warmer temperatures of 18–19 °C have been shown to exhibit reduced thyroxine (T_4) concentrations, higher T_4 conversion to 3,5,3′-triiodo-$_L$-thyronine (T_3), and elevated rates of outer-ring TH deiodination in liver microsomes (Eales et al., 1982; Johnston and Eales, 1995). While THs are well established mediators of metabolism and development in fish and other vertebrates, THs also influence gonad development and function (Jannini et al., 1995; Dittrich et al., 2011; Habibi et al., 2012; Flood et al., 2013; Sharma and Patiño, 2013; Castañeda Cortés et al., 2014; Duarte-Guterman et al., 2014). Such associations between TH status and reproduction have been recognized for some time in teleost fishes, although studies are only now uncovering the complexity of those interactions (Cyr and Eales, 1996; Matta et al., 2002; Raine, 2011; Habibi et al., 2012; Tovo-Neto et al., 2018; Feng et al., 2022). In adult *Clarias gariepinus* catfish, for instance, goitrogen treatment reduced testicular T concentrations and led to testes containing fewer spermatids and spermatozoa (Jacob et al., 2005). And in goldfish (*Carassius auratus*), T_3 both reduced gene expression of LH β-subunit (*lhβ*) in cultured pituitary cells and lowered circulating concentrations of E_2 in vivo in males (Allan and Habibi, 2012). The picture emerging from those and other studies suggests that TH interactions with the HPG axis in fish are complex and condition-dependent (Nelson et al., 2010; Tovo-Neto et al., 2018; Deal and Volkoff, 2020; Ma et al., 2020a). Recent studies with female goldfish observed that T_3 interacts with both GnRH and gonadotropin-inhibitory hormone (GnIH) to modulate LH release in differing ways depending on stage of the reproductive cycle (Ma et al., 2020b). Such TH actions on reproductive function also appear to extend to tissues involved in reproduction but beyond the HPG axis directly. In goldfish females – but not males – T_3 increased hepatic Vtg mRNA levels, an effect hypothesized to be mediated by T_3 modulation of hepatic estrogen receptor α (*esr1*) gene expression (Nelson and Habibi, 2016). Even so, while the roles of THs in fish reproduction are becoming more well-defined, it still is not known if temperature-associated changes in TH signaling contribute to reproductive inhibition in fish experiencing elevated temperatures.

In the present study, we examined interactions between temperature and TH status on gene pathways linked to reproductive function in Amargosa pupfish, (*Cyprinodon nevadensis amargosae*). Pupfishes of genus *Cyprinodon* evolved some of the highest temperature tolerances of fish (Beitinger et al., 2000; Bennett and Beitinger, 1997; Feldmeth, 1981; Minckley and Minckley, 1986; Heath et al., 1993; Nordlie, 2006; Carson et al., 2014; Motani and Wainwright, 2015). Many pupfishes survive in habitats where temperatures routinely reach 40 °C or greater, and some members of this genus even inhabit environments with temperatures exceeding 44 °C (e.g., Minckley and Minckley, 1986; Carson et al., 2014). Still, environmental temperature plays an important role in regulating pupfish reproductive activity (Barlow, 1961; Liu, 1969; Echelle, 1973). The species studied here – the Amargosa pupfish – has the highest experimentally demonstrated reproduction temperature of any fish species examined to date (Motani and Wainwright, 2015; see also Shrode and Gerking, 1977). While spawning by Amargosa pupfish can occur in the wild at any time of the year when temperatures rise above ~18 °C (Soltz, 1974), the optimal temperatures for egg production by Amargosa pupfish ranges from 24 °C to 32 °C (Shrode and Gerking, 1977). Above ~32 °C, egg quality of Amargosa pupfish declines such that a greater proportion of eggs are produced either lacking yolk or with soft, underdeveloped chorions (Shrode and Gerking, 1977; Gerking et al., 1979). And, above ~34 °C, reproduction by *C. nevadensis* may cease (Shrode and Gerking, 1977; Gerking and Lee, 1983), despite that temperature being considerably lower than the CT_{max} value of ~42 °C for the species (Brown and Feldmeth, 1971; Feldmeth et al., 1974). Climate models predict increasing temperatures in the deserts of the North American Southwest where this and other pupfish species live (Seager et al., 2007; Seager and Vecchi, 2010), and understanding the mechanisms by which temperature influences reproductive performance in pupfish and other fish species will be critical for identifying which populations are most likely to survive under a changing climate (Chown et al., 2010; Hoffman and Sgrò, 2011).

Here, we examined the effects of temperature and T_3 on reproductive endocrine signaling and gametogenesis in *C. n. amargosae* pupfish obtained from two allopatric populations: the Amargosa River and Tecopa Bore (Fig. 1). The Amargosa River is small desert stream with highly variably environmental conditions (Soltz and Naiman, 1978). Temperatures in this habitat vary from near freezing to over 40 °C seasonally and can shift as much as 25 °C between day and night (Lema et al., 2019). Tecopa Bore is a small artesian spring where water emerges from the ground at 47.5 °C and then gradually cools as it flows through a marsh (Naiman, 1974; Lema et al., 2019). The Tecopa Bore habitat was

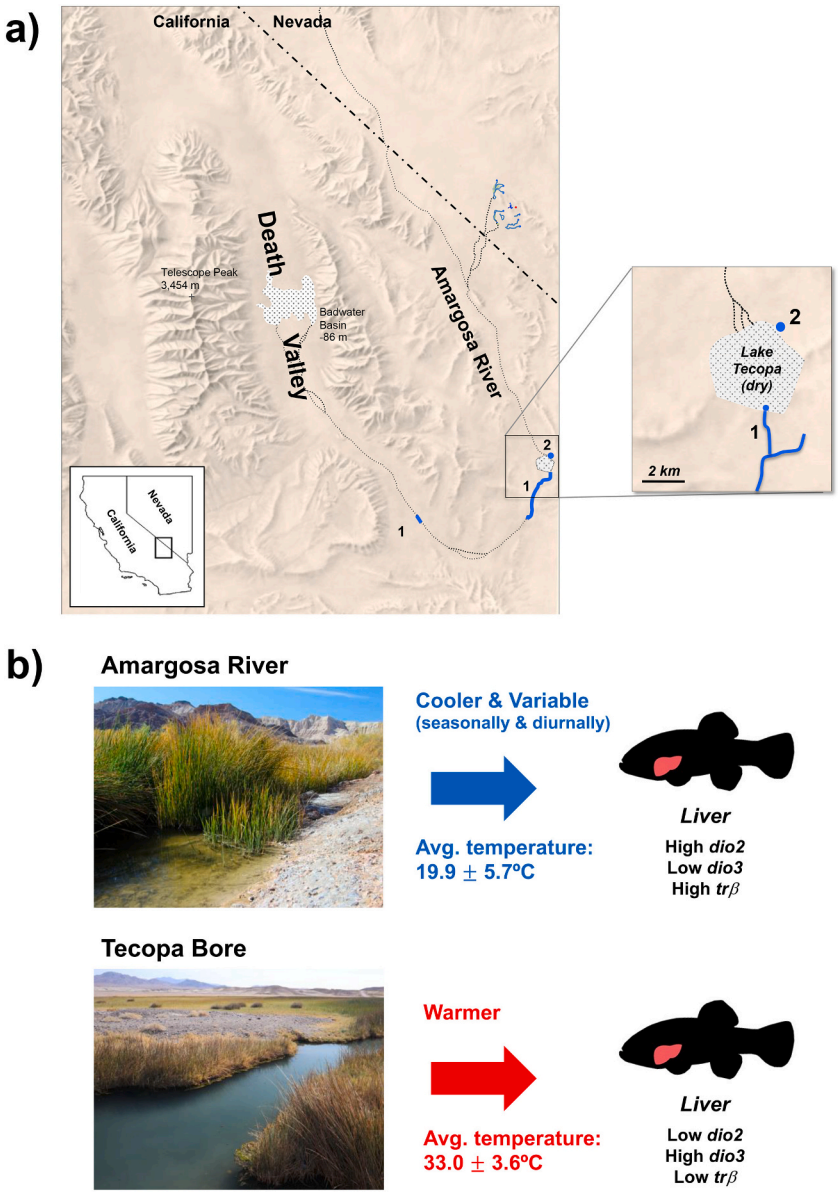


Fig. 1. (a) Map showing locations of the Amargosa River and Tecopa Bore habitats for the Amargosa pupfish (*Cyprinodon nevadensis amargosae*) in the Death Valley region of California and Nevada, USA. Numbers on the map denote locations of the following: (1) the Amargosa River, which is dry along most of its length except during extreme rainfall events, but has two groundwater-fed permanent reaches – one longer ~13 km permanent section beginning near the dry lakebed of former Lake Tecopa, and a smaller section of permanent water in the river's lower basin; and (2) Tecopa Bore, a small marsh habitat fed by a hot temperature groundwater spring on the north-eastern edge of dry Lake Tecopa. (b) The Amargosa River is a variable temperature stream where, on average, temperatures are cooler with both diurnal and seasonal variation. Tecopa Bore is a marsh habitat fed by a hot spring. Both habitats support allopatric populations of *C. n. amargosae* that differ both morphologically and physiologically. Those physiological differences include variation in thyroid hormone status with pupfish from the warmer Tecopa Bore habitat both in the wild and under common laboratory conditions having higher type 3 iodothyronine deiodinase (*dio3*), lower type 2 iodothyronine deiodinase (*dio2*), and lower thyroid hormone receptor β (*trβ*) mRNA expression in the liver (Lema et al., 2016). Habitat temperatures provided are mean ± SD values, as reported by across March 2014 to February 2016 by Lema et al. (2019). Map modified from Lema (2014).

created only ~55 years ago, when mineral drilling by a private company created the artesian spring; the newly formed Tecopa Bore environment was soon after colonized by *C. n. amargosae* from the nearby Amargosa River. In the wild, *C. n. amargosae* from the Amargosa River and Tecopa Bore populations differ in liver gene expression for type 2 (*dio2*) and type 3 (*dio3*) iodothyroine deiodinase enzymes and thyroid hormone receptor β (*trβ*) (Fig. 1) (Lema et al., 2016). Those differences in liver TH signaling gene expression sustained even after fish from the populations were held under common temperature conditions in the laboratory, and link to broader differences in physiology and phenotypic development between the populations (Lema et al., 2016, 2019; Lema, 2020). In the present study, sexually-mature female and male pupfish from the Amargosa River and Tecopa Bore populations were acclimated to stable conditions of 24 °C or 34 °C for 88 days and then treated with exogenous T_3 in order to: 1) to test how elevated temperature and T_3 interact to affect gene expression pathways associated with HPG axis signaling and gametogenesis, and 2) to determine whether the Amargosa River and Tecopa Bore pupfish populations show differential T_3 effects on liver expression of reproduction-associated genes regulated by E_2 action, given previously observed differences in liver TH signaling between the populations (Lema et al., 2016).

2. Materials and methods

2.1. Animals

Adult *C. n. amargosae* pupfish were collected by minnow trap on 9 May 2014 from two habitats located in the Death Valley desert region of eastern California, USA: the Amargosa River (35°51.275′N, 116°13.833′W) and Tecopa Bore (35°53.140′N, 116°14.050′W). Details on the environmental differences between these habitats as well as the phenotypic differences between the Amargosa River and Tecopa Bore *C. n. amargosae* populations are provided elsewhere (Lema et al., 2016, 2019; Lema, 2020). Pupfish were transported to California Polytechnic State University in San Luis Obsipo, California, USA, and maintained in 208 L tanks. For both the initial holding period and during experimental testing, all fish were maintained under a 14 L:10D photoperiod in synthetic 2.1 ppt salinity water made with Instant Ocean® salt (Unified Pet Group, Inc., Blacksburg, VA, USA) and deionized water. Pupfish were acclimated to captivity at temperatures representative of their

respective habitats: 24 °C for pupfish from the Amargosa River, and 34 °C for fish from Tecopa Bore. These temperatures were selected to compare a temperature within the optimal range for reproductive performance (24 °C) for this species to an elevated temperature (34 °C) shown to impair egg quality and spawning (e.g., Shrode and Gerking, 1977; Gerking et al., 1979; Feuerbacher et al., 2015). All experimental procedures were approved by the Animal Care and Use Committee of California Polytechnic State University (Protocol #1507).

Within 16 d of collection, pupfish were transferred to 38 L experimental tanks with 2 males and 2 females per tank. Four replicate tanks were used for each temperature-population-hormone treatment combination. The average body size of females (37.57 ± 1.01 mm standard length [SL], 1.53 ± 0.12 g mass; mean ± SEM) and males (40.96 ± 0.85 mm SL, 2.18 ± 0.12 g mass) from the Amargosa River was larger than that of females (30.97 ± 0.55 mm SL, 0.82 ± 0.05 g mass) and males (38.85 ± 0.67 mm SL, 1.88 ± 0.10 g mass) from Tecopa Bore (SL: population*sex interaction, $F_{1,116} = 8.067$, $p = 0.0053$; mass: population effect, $F_{1,116} = 22.416$, $p < 0.0001$), which reflects previously documented body size differences between these populations (Lema et al., 2019). Pupfish were maintained in these 38 L tanks for 88 days under stable temperatures of 24 °C or 34 °C. Temperature was recorded at 10 min intervals using HOBO® U12 External Data Loggers (Onset Corp., Bourne, MA, USA), which confirmed average temperatures in the treatments to be 23.97 ± 0.40 °C and 34.18 ± 0.24 °C (mean ± SD). All fish were fed ad lib twice daily with a 1:1 ratio mixture of commercial spirulina flakes (Aquatic Eco-Systems, Inc., Apopka, FL) and brine shrimp flakes (San Francisco Bay Brand, Inc., Newark, CA) for the duration of the experiment.

On the day immediately prior to sacrifice and tissue collection, fish in a subset of tanks were exposed to a 15 nM concentration of waterborne 3′-triiodo-$_L$-thyronine (T_3) for 18–24 h. The exogenous T_3 treatment was generated by dissolving T_3 (Sigma-Aldrich) in 0.01 M NaOH and adding stock T_3 solution to a tank; all other tanks received control vehicle (0.01 M NaOH) only. Additional data on variation in TH signaling and metabolic physiology measured from the same fish used in this same experiment are reported in Lema et al. (2016).

2.2. Tissue collection

Fish sampling from the experimental 38 L tanks occurred between the hours of 13:00 and 16:00 to minimize photoperiod influences. Each fish was collected by hand net and euthanized (tricaine methansulfonate, MS222; Argent Chemicals, Inc., Redmond, WA, USA). Fish were measured (SL, ± 0.05 mm) and weighed (± 0.01 g), and blood was collected. Blood was centrifuged for 10 min at 3000 rpm and 4 °C, and the resulting plasma stored at −80 °C for subsequent T_3 quantification by radioimmunoassay following the procedure of Dickhoff et al. (1982). Anti-L-T_3 antiserum (1:10,000) for this RIA was obtained from Fitzgerald Industries International (Acton, MA, USA). The intra-assay % coefficient of variation (CV) for the T_3 RIA was 4.83%, and inter-assay CV was 6.21%. Specific details on these RIA methods are provided in Lema et al. (2016). The gonads and liver of each fish were also dissected, flash frozen with liquid N_2, and stored at −80 °C.

2.3. Quantitative real-time RT-PCR

Total RNA was extracted from gonad and liver tissues using Tri-Reagent (Molecular Research Center, Inc.) with bromochloropropane as the phase separation reagent. The resulting RNA was DNase I treated (TURBO DNA-free Kit, Ambion) and quantified using a P300 NanoPhotometer (Implen, Inc.) (260:280 ratios >1.97). Total RNA was reverse transcribed in 20 µl reaction volumes containing 6 µl of RNA (100 ng/µl for liver or 33 ng/µl for gonad), 1.5 µl dNTPs (Promega), 1.5 µl random primers (500 µg/ml; Promega), 0.375 µl recombinant RNasin ribonuclease inhibitor (40 u/µl; Promega), 9 µl nuclease-free H_2O, and 6 µl 5× buffer, 4.5 µl $MgCl_2$, and 1.125 µl GoScript™ reverse transcriptase (Promega). Reverse transcription reactions were run as the following thermal profile: 25 °C for 5 min and 42 °C for 1 h, followed by 70 °C for 15 min to inactivate the reverse transcriptase.

Primer sets were designed to quantify relative expression levels of several genes linked to reproductive endocrine function in the gonads including a follicle-stimulating hormone receptor (*fshr*) and luteinizing hormone/choriogonadotropin receptor (*lhcgr*), the steroidogenic enzymes ovarian cytochrome P450 aromatase (*cyp19a1a*), 3β-hydroxysteroid dehydrogenase (*3βhsd*), 11β-hydroxysteroid dehydrogenase (*11βhsd*), and 17β-hydroxysteroid dehydrogenase (*hsd17β3*), and the steroid acute regulatory protein (*star*) transporter (Supplemental Materials, Table S1). Transcript abundances for nuclear estrogen receptors *esr1, esr2a* and *esr2b*, vitellogenins A (*vtgA*) and B (*vtgB*), and choriogenins L (*cgL*), H (*cgH*), and H-minor (*cgHm*) were also quantified in the liver. Primer sets for SYBR Green qRT-PCR assays were designed to protein coding regions of partial cDNAs for choriogenins *cgL* (GenBank accession no. KJ850333) and *cgHm* (KJ850332) sequenced from *C. n. amargosae* using degenerate primers described previously (Johnson and Lema, 2017). All other qRT-PCR assays were designed to genes identified from the unannotated genome of the closely-related pupfish *C. n. pectoralis* (GenBank accession no. GCA_000776015). When possible, qRT-PCR primer sets were designed to span an intron boundary. Primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA), and nucleotide sequences for all primers are provided in Supplemental Materials, Table S1. The specificity of each primer set was confirmed by Sanger sequencing select PCR amplification products amplified by each primer set. Relative mRNA levels for elongation factor-1α (*ef1α*) were quantified as a control gene using SYBR green primers designed and validated previously (Lema, 2010; Lema et al., 2015).

All qRT-PCR reactions were run as 16 µl reaction volumes containing 8.0 µl iQ Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 0.8 µl each of forward and reverse primers (10 µM), 4.9 µl nuclease-free water (Sigma, St. Louis, MO, USA), and 1.5 µl of reverse-transcribed cDNA template. Assays were run on either a 7300 Real-Time PCR System (Applied Biosystems, Inc.) or CFX Connect Real-Time PCR System (Bio-Rad) with the following thermal profile: 50 °C for 2 min, 95 °C for 10 min, 42 cycles of 95 °C for 15 s and 59 °C for 1 min, followed by a melt curve analysis. For each gene, a standard curve was made from a pool of RNA from samples representing all factor categories (e.g., population, temperature regime, hormone treatment, sex). Each standard was serially diluted and assayed in triplicate. DNA contamination was assessed for each gene by analyzing RNA samples that were not reverse-transcribed. Each qPCR run also included samples without cDNA template as a further contamination control. PCR efficiencies for each gene were calculated using the equation: % efficiency = $[10(^{1/slope}) - 1]\bullet 100$, and are provided in Supplemental Materials, Table S1. Correlation coefficients (r^2) were < 0.97 for the standard curve for each gene examined. The expression of each gene of interest was expressed as a relative level by dividing the resulting values by the mean values of females in the lower 24 °C temperature, control injection treatment group. The mean intra-assay coefficient of variation (CV) was 7.17%, and inter-plate CV was calculated as 9.22%.

2.4. Statistical analyses

Plasma T_3 concentrations were $\log_{10}$ transformed to meet parametric assumptions and then compared using a three-factor ANOVA model with population origin, temperature, and hormone treatment as factors. All interaction terms were included in the ANOVA model. Sexes were combined for plasma T_3 analyses since the small number of females in some population-temperature-hormone treatment combinations precluded the analysis of treatment effects on sexes separately.

Preliminary statistical analyses revealed that gonadal *ef1α* mRNA abundance varied significant between the sexes ($p < 0.001$). Statistical analyses on gonadal gene transcript abundance were therefore conducted separately for female and male pupfish. Even so, data values for

female and male gonadal mRNA levels were normalized to the same scale to allow for visual comparisons of sex differences in gonadal transcript abundance on figures. Gene transcript data for the ovary and testis were $\log_{10}(x + 1)$ transformed, and then analyzed using ANOVA models with population origin, temperature, and hormone treatment as main effect factors and all interaction terms. Post hoc Tukey HSD tests were then calculated to identify pairwise differences among treatment groups.

The abundance of gene transcripts encoding *vtgA, vtgB, cgL*, *cgH*, and *cgHm* in the liver were examined in females only since cDNA amplification of these transcripts using qRT-PCR was either undetectable or detected at exceedingly low abundance in male fish. Transcripts encoding the nuclear ER genes *esr1*, *esr2a,* and *esr2b* however, were examined in both sexes separately. Hepatic transcript data were also $\log_{10}(x + 1)$ transformed and analyzed using ANOVA models with population origin, temperature, and hormone treatment as factors as well as all interaction terms, as described above. To further explore interactions between liver estrogen pathway-associated gene expression and thyroid hormone status, principal components analysis (PCA) was used to test for associations among patterns of gene transcript regulation in the liver. Principal components (PCs) with eigenvalues <1.5 were then used for further analyses. Analysis of covariance (ANCOVA) models were used to test whether individual fish PC values varied in patterns associated with population origin, temperature treatment, hormone injection treatment. Data for liver type 2 iodothyronine deiodinase (*dio2*), type 3 iodothyroine deiodinase (*dio3*), and thyroid hormone receptor β (*trβ*) mRNA abundance reported in Lema et al. (2016) were from the same individual pupfish used in the current experiment; those relative mRNA values were thus again used here in ANCOVA models to test for associations between liver PC2 and liver *dio2, dio3*, and *trβ* mRNA levels.

All data are presented as mean ± SEM values, and all statistical tests were two-tailed. All statistical analyses were calculated using JMP Pro 15 software (SAS Institute, Inc.).

3. Results

3.1. Temperature effects on gene pathways linked to steroidogenesis and oogenesis

Males and females from both populations exhibited similar temperature-induced responses of gonadal *fshr* and *lhcgr* mRNA expression. The relative abundance of *fshr* transcripts was significantly reduced in both the ovary (temperature effect: $F_{1,40} = 5.486$, $p = 0.0242$) and testes ($F_{1,44} = 7.765$, $p = 0.0078$) of fish held at 34 °C compared to 24 °C, while only males exposed to the higher temperature exhibited a significant reduction ($F_{1,44} = 5.658$, $p = 0.0218$) in relative *lhcgr* expression (Fig. 2).

The most significant differences in steroidogenic gene expression in response to temperature variation occurred in Tecopa Bore females. Compared to those held at 24 °C, Tecopa Bore females at 34 °C produced significantly less mRNA transcripts for *3βhsd* (temperature effect: $F_{1,40} = 6.985$, $p = 0.0117$), *hsd17β3* (temperature effect: $F_{1,40} = 5.1824$, $p = 0.0282$), and *cyp19a1a* (population*temperature interaction, $F_{1,40} = 6.1594$, $p = 0.0174$) (Fig. 3). Relative expression levels for *star* mRNAs also trended toward being reduced in Tecopa Bore females at 34 °C, although the effect was not significant (temperature*population effect: $F_{1,40} = 4.018$, $p = 0.0518$) (Fig. 3a). Ovarian expression of *11βhsd* in Tecopa Bore fish, however, did not vary with temperature (Fig. 3d). Temperature was not a significant factor affecting steroidogenic gene expression in the ovaries of Amargosa River females or in the testes of males from either population.

Similar to the steroidogenic gene expression, hepatic abundances of transcripts encoding vitellogenin and choriogenin genes exhibited high temperature-induced reductions in Tecopa Bore but not Amargosa River females (Fig. 4a,b) (*vtgA*: population*temperature interaction: $F_{1,47} = 9.690$, $p = 0.0032$; *vtgB*: population*temperature interaction: $F_{1,47} = 5.028$, $p = 0.0269$). Hepatic gene transcripts encoding choriogenins *cgL* (population*temperature interaction: $F_{1,47} = 7.225$, $p = 0.0099$), *cgH* (population*temperature interaction: $F_{1,47} = 4.297$, $p = 0.0437$), and *cgHm* (population*temperature interaction: $F_{1,47} = 7.406$, $p = 0.0091$) were all also higher in Tecopa Bore females at 24 °C than at 34 °C (Fig. 4c-e).

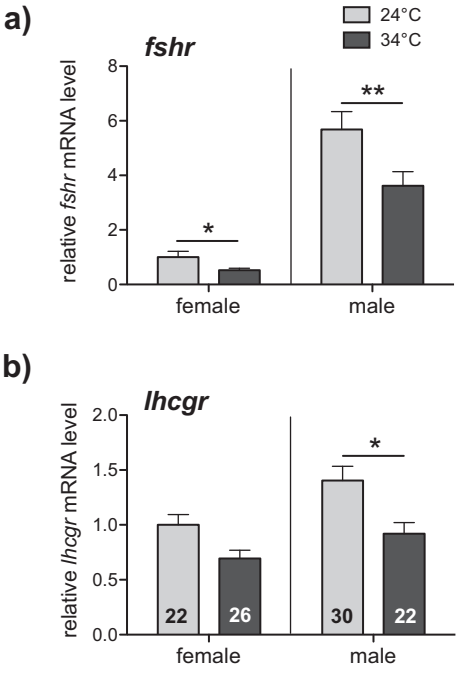


Fig. 2. Temperature-induced changes in levels of gonadal gene transcripts for FSH receptor (*fshr*) and LH receptor (*lhcgr*). (a) Relative mRNA levels for *fshr* were lower in the ovary and testis of pupfish at 34 °C compared to 24 °C. (b) Male pupfish at 34 °C also exhibited lower relative gene transcript abundance for *lhcgr* in testis, although no statistical effect of temperature was detected for ovarian *lhcgr* expression. Pupfish originating from both the Amargosa River and Tecopa Bore populations responded similarly to temperature, so data from both populations are plotted together. Sample sizes (*n*) for each sex and temperature treatment are provided in (b). Asterisks indicate post hoc pairwise comparisons wherein * indicates $p < 0.05$ and ** indicates $p < 0.025$.

Hepatic *esr1* transcripts were less abundant in female pupfish at 34 °C than at 24 °C (Fig. 5a; temperature effect: $F_{1,47} = 9.787$, $p = 0.0030$). In male pupfish, hepatic *esr1* mRNA levels were also affected by temperature, although the direction of that effect differed between the populations (population*temperature interaction: $F_{1,56} = 7.121$, $p = 0.0099$) such that only males from the Tecopa Bore habitat exhibited reduced hepatic *esr1* mRNA levels at 34 °C. Hepatic mRNAs encoding *esr2a* were at lower relative levels in both females and males at 34 °C than at 24 °C (Fig. 5b) (temperature effect, females: $F_{1,47} = 12.348$, $p = 0.0010$; males: $F_{1,56} = 4.065$, $p = 0.0486$). Liver *esr2b* mRNA levels were similarly reduced in males experiencing the higher 34 °C temperature (temperature effect: $F_{1,56} = 9.110$, $p = 0.0039$), although post hoc pairwise comparison revealed this temperature effect was more pronounced in males from the Amargosa River than from Tecopa Bore (Fig. 5c). For females, however, liver *esr2b* mRNA abundance was only reduced at 34 °C in females from the Amargosa River and not those from Tecopa Bore (temperature*population interaction: $F_{1,47} = 22.907$, $p < 0.0001$).

3.2. T_3 effects on steroidogenic enzyme and estrogen receptor gene expression

Treatment with exogenous T_3 increased plasma T_3 concentrations in fish from both populations, however, the resulting plasma T_3

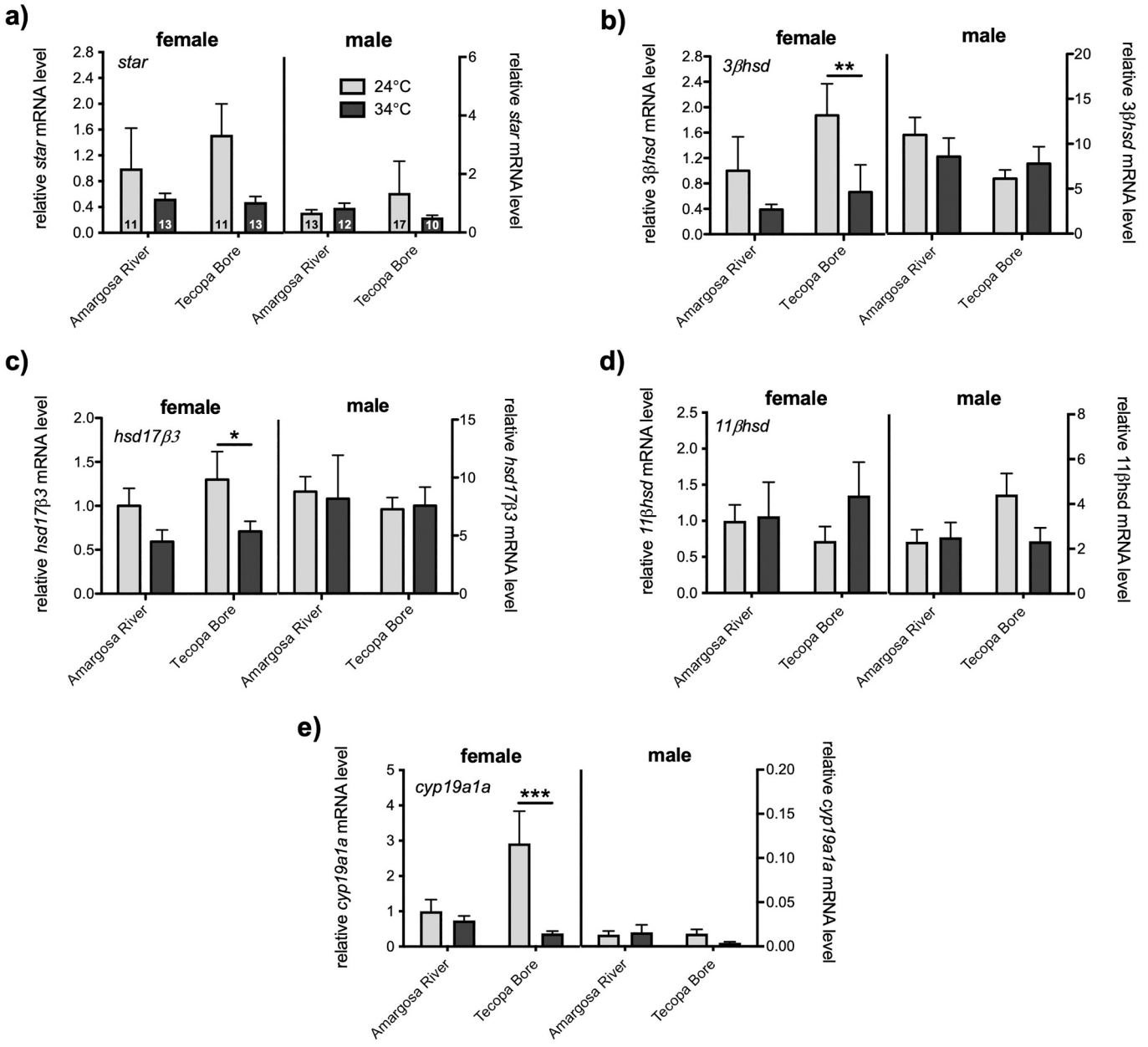


Fig. 3. Temperature influences on steroid acute regulatory protein (*star*) and steroidogenic enzyme gene transcript levels in the gonads. Transcripts encoding (a) *star* were marginally lower in the gonad of female at the higher 34 °C than at 24 °C. Transcript abundances for (b) *3βhsd* and (c) *hsd17β3* were both at lower relative abundance in the ovary of Tecopa Bore pupfish at 34 °C than at 24 °C. (d) Gonadal *11βhsd* mRNA levels did not vary in either sex. (e) Female pupfish from Tecopa Bore maintained at 34 °C had lower relative *cyp19a1a* mRNA levels in the ovary compared to females at 24 °C. This temperature effect was only observed in females from the Tecopa Bore population and not in the Amargosa River. Sample sizes (*n*) for each group are provided in (a). Asterisks indicate: * $p < 0.05$, ** $p < 0.025$, and *** $p < 0.0001$.

concentration of pupfish at 34 °C was over three-fold higher than that of 24 °C fish (temperature*hormone interaction: $F_{1,61} = 8.732$, $p = 0.0044$) (Table 1). While we are unaware of any study that has examined temperature-dependent kinetics of T_3 uptake from water in fish; waterborne E_2 uptake across the fish gill has been shown to be associated positively with temperature (Blewett et al., 2013a, 2013b), and temperature-associated variation in uptake rates likely helps explain the high plasma T_3 concentrations observed in hormone-treated pupfish from the 34 °C treatment.

Treatment with T_3 increased the abundance of *star* transcripts in the testis (hormone effect: $F_{1,44} = 6.202$, $p = 0.0166$), with a more pronounced hormone effect in males from the Amargosa River than from Tecopa Bore (Fig. 6a). Conversely, in females, the effect of T_3 varied significantly by population (population*hormone interaction: $F_{1,40} = 4.663$, $p = 0.0369$), with Tecopa Bore females exhibiting a greater T_3-mediated increase in ovarian *star* transcript levels than Amargosa River females (Fig. 6a). Male pupfish from the Amargosa River – but not from Tecopa Bore – treated with T_3 at both acclimation temperatures had a lower relative abundance of testicular *hsd17β3* transcripts (Fig. 6b) (population*hormone interaction: $F_{1,44} = 7.807$, $p = 0.0077$). Females from both populations expressed similar levels of *hsd17β3* in the absence of any effect of T_3.

T_3 also altered hepatic *esr1* estrogen receptor mRNAs in both female and male pupfish from both populations. In males, the effect of T_3 on hepatic *esr1* mRNA levels depended on temperature (temperature*hormone interaction: $F_{1,56} = 19.784$, $p < 0.0001$), with T_3-treated

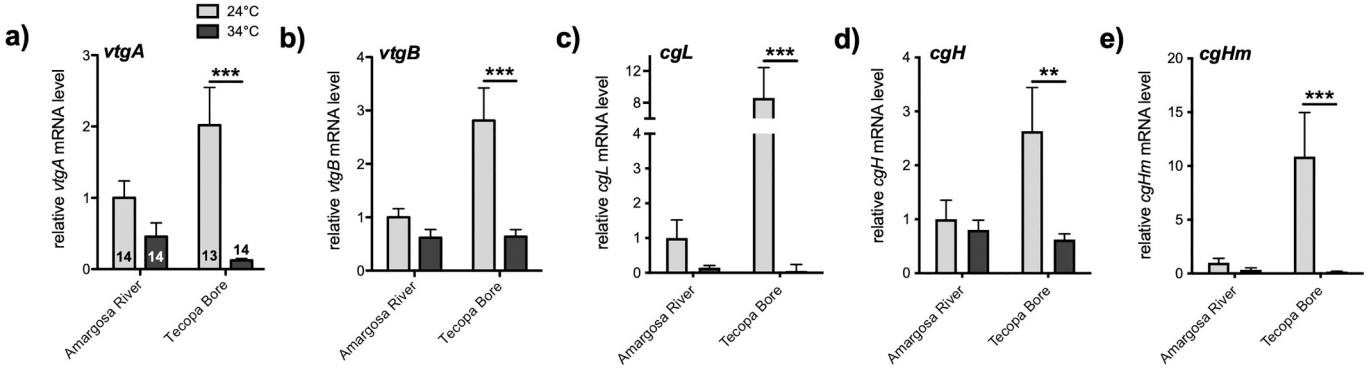


Fig. 4. Hepatic mRNA abundance for the vitellogenins *vtgA* (a) and *vtgB* (b) and the choriogenins *cgL* (c), *cgH* (d), and *cgHm* (e) was significantly higher in female pupfish from Tecopa Bore population maintained at 24 °C compared to females at 34 °C. Those temperature effects were only detected in females from Tecopa Bore, and not from the Amargosa River. Sample sizes (*n*) for each treatment are provided in (a). Asterisks indicate post hoc pairwise comparisons: ** $p < 0.025$ and *** $p < 0.0001$.

males expressing higher relative levels of hepatic *esr1* at 24 °C, but lower *esr1* mRNA abundance following T_3 supplementation at 34 °C (Fig. 7a). While the overall ANOVA indicated that the direction of effects of T_3 was consistent across populations, post hoc pairwise comparisons indicated the amount of T_3-induced decline in *esr1* transcript varied somewhat among populations. In females, *esr1* expression was decreased in fish treated with T_3 (hormone effect: $F_{1,48} = 5.299$, $p = 0.0257$) (Fig. 7b), although the extent of this decline was relatively small and not statistically significant in *post hoc* pairwise comparisons. T_3 also reduced hepatic *esr2a* mRNA levels in female pupfish with that effect most pronounced in Tecopa Bore females at 34 °C (Fig. 7c) (hormone effect: $F_{1,47} = 7.245$, $p = 0.0098$). T_3 had no effect on liver *esr2a* transcript abundance in males. T_3 did not affect hepatic *esr2b* mRNA levels in either sex.

Principal component analysis on liver estrogen-associated pathway mRNA levels in females generated two PCs that together accounted for 78.4% of the variation in hepatic mRNA profiles (Fig. 8a). PC1 (eigenvalue = 4.274) explained 53.4% of the variation in liver mRNA abundance with transcripts encoding vitellogenins *vtgA* and *vtgB* and choriogenins *cgL, cgH*, and *cgHm* all loading positively on PC1 (Fig. 8b). PC2 (eigenvalue = 2.001) explained another 25.0% of the observed variation in hepatic mRNA levels. Transcripts encoding the three measured estrogen receptors – *esr1, esr2a,* and *esr2b* – all had high positive loadings on PC2 (Fig. 8b). Comparison of PC values across population, temperature, and T_3 treatment conditions revealed effects similar to those observed when transcript abundances were analyzed as individual genes (Fig. 8c). PC1 values, comprising transcripts for vitellogenin and choriogenins, exhibited a temperature effect that depended on population (population*temperature interaction: $F_{1,47} = 7.547$, $p = 0.0085$). Only Tecopa Bore females held at the lower temperature of 24 °C had elevated transcripts associated with PC1. Temperature did not affect PC1 values in Amargosa River females, and there was no effect of T_3 on PC1 for either population or temperature treatment. For PC2, the effects of temperature again varied depending on population origin (population*temperature interaction: $F_{1,47} = 14.491$, $p = 0.0004$). Liver PC2 values were greatly reduced in Amargosa River females at 34 °C but showed only minor reductions for Tecopa Bore females at that same elevated temperature. T_3 reduced liver PC2 values (hormone effect: $F_{1,47} = 5.189$, $p = 0.0273$), although this effect appeared considerably more pronounced in females from the Tecopa Bore population (Fig. 8c).

TH effects on liver vitellogenin expression have been shown to occur via TH upregulation of estrogen receptor *esr1* via a thyroid hormone receptor β (*trβ*) pathway (Nelson and Habibi, 2016). Since prior work documented persistent differences in liver *trβ* expression, as well as liver *dio2* and *dio3* iodothyronine deiodinase expression, between pupfish females from the Tecopa Bore and Amargosa River populations (Lema et al., 2016), we also tested for relationships between liver PC2 values and liver *trβ*, *dio2*, and *dio3* expression to explore whether observed population differences in PC2 values might link to liver TH pathway gene expression. These analyses revealed that individual differences in liver PC2 related positively to individual variation in liver mRNA abundance for both *trβ* ($F_{1,39} = 9.089$, $p = 0.0045$) and *dio2* ($F_{1,39} = 7.467$, $p = 0.0094$) in both populations (Fig. 8d). Liver PC2 also showed a relationship with liver *dio3* mRNA abundance wherein females with elevated *dio3* due to T_3 treatment exhibited lower PC2 values (hormone*liver *dio3* mRNA level interaction: $F_{1,39} = 5.425$, $p = 0.0251$).

4. Discussion

Pupfishes of the southwestern deserts of North America evolved to tolerate some of the hottest temperature environments inhabited by Actinopterygiian fishes (Brown and Feldmeth, 1971; Feldmeth et al., 1974; Motani and Wainwright, 2015), and yet these species – like other fishes – still show reductions in reproductive output at high temperatures (e.g., Shrode and Gerking, 1977; Feuerbacher et al., 2015; Bock et al., 2021). Here, we examined how temperature conditions influence the relative abundance of transcripts encoding genes key to reproductive endocrine signaling in the gonad or liver (e.g., Nakamura et al., 2005; Kusakabe et al., 2006), with the aims of examining whether changes in HPG endocrine signaling might help explain the reduced reproductive performance observed in pupfish at high temperature and testing whether conspecific populations from different thermal conditions in the wild vary in those gene expression responses to temperature. We also tested for effects of T_3 modulation of gametogenic and steroidogenic pathways under those differing temperature conditions to explore how variation in TH signaling might contribute to alterations in reproductive performance at elevated temperatures.

4.1. High temperature effects on HPG axis and oogenesis pathways

Data obtained in this study showed that sexually-mature female and male *C. n. amargosae* pupfish exposed to a stable 34 °C environment had several changes in HPG axis gene expression that correspond with what has been observed previously in *Cyprinodon* pupfish at elevated temperature (Bock et al., 2021), as well as other teleost fishes experiencing high temperatures (e.g., Alix et al., 2020). Those changes include a lower abundance of transcripts encoding receptors for FSH and LH in the gonads. While we were unable to histologically evaluate the stages of oogenesis and spermatogenesis in the gonadal tissues of pupfish in the current study, that female and male *C. n. amargosae* maintained at 34 °C show a lower gonadal abundance of *fshr* and *lhcgr* transcripts suggests that pupfish at this elevated temperature may have altered gonadotropin stimulation of the gonad (Planas and Swanson, 2008; Levavi-Sivan et al.,

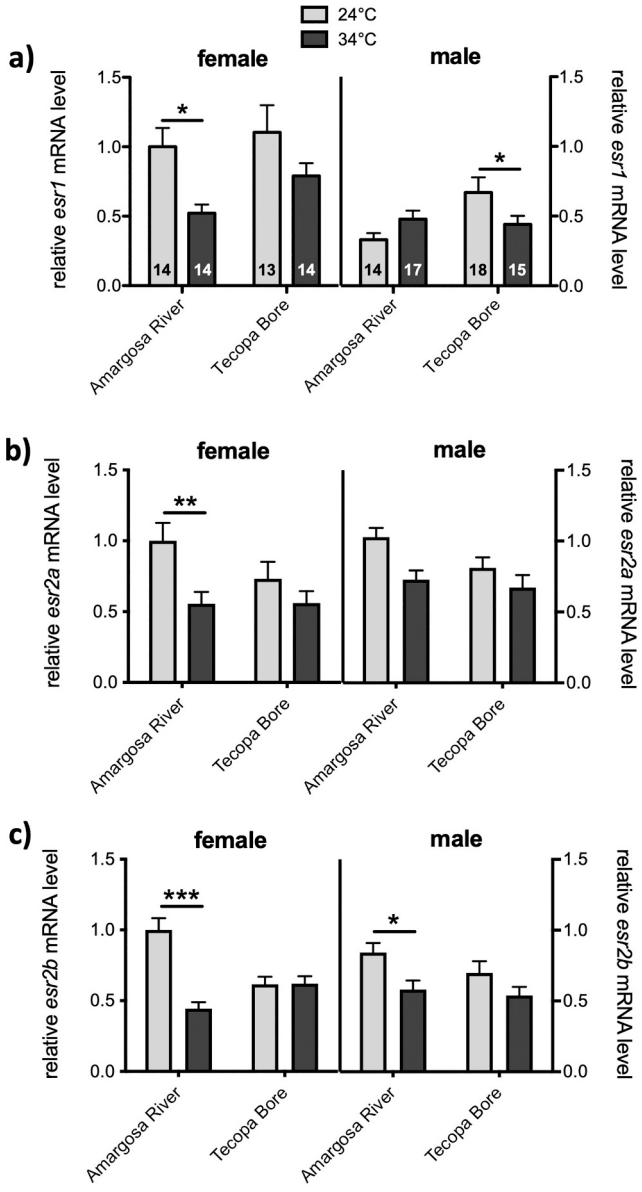


Fig. 5. Liver mRNAs encoding estrogen receptors (a) *esr1*, (b) *esr2a*, and (c) *esr2b* were at lower relative levels in females maintained at 34 °C temperature than at 24 °C. Pairwise post hoc comparisons revealed these temperature effects on hepatic estrogen receptor mRNA levels to be more pronounced in females from the Amargosa River habitat than from Tecopa Bore. Males from Tecopa Bore, but not the Amargosa River, likewise exhibited lower hepatic *esr1* transcript abundance at 34 °C. Hepatic *esr2b* receptor mRNA expression was also reduced in the testis of Amargosa River males at 34 °C. Sample sizes (*n*) are provided in (a), and asterisks indicate post hoc comparisons: * $p < 0.05$, ** $p < 0.025$, and *** $p < 0.001$.

Table 1
Plasma T_3 concentration (mean ± SEM) in pupfish acclimated to 24 °C or 34 °C and treated with waterborne T_3.

Acclimation temperature	Hormone treatment	Plasma T_3 (ng/ml)
24 °C	control	12.98 ± 2.59^{a}
	T_3	94.96 ± 11.91^{b}
34 °C	control	12.72 ± 1.38^{a}
	T_3	328.45 ± 38.01^{c}

Note: Letters indicate Tukey HSD comparisons among groups.

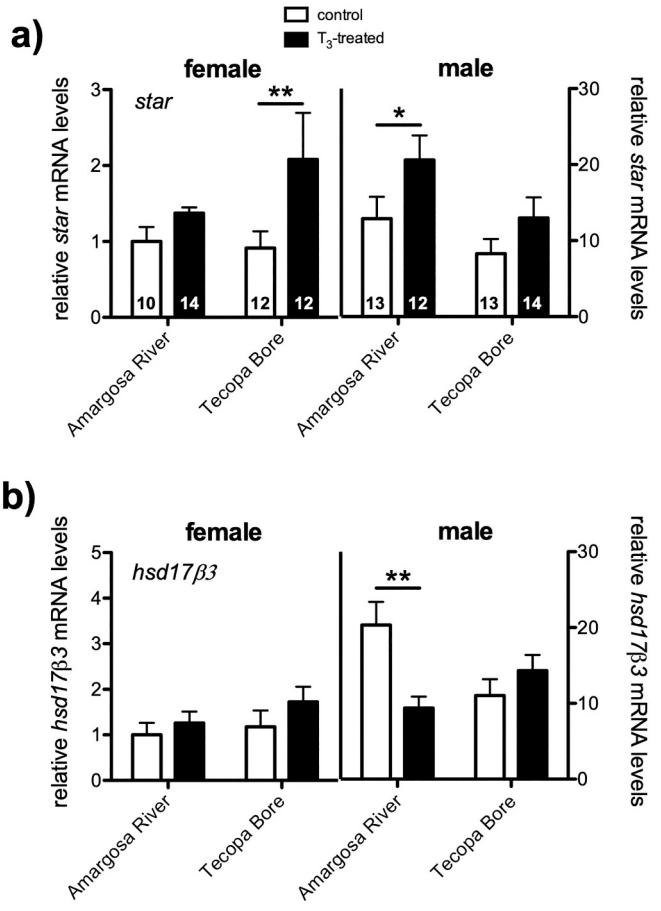


Fig. 6. T_3 affects gonadal mRNA levels for (a) *star* and (b) *hsd17β3*. Sample sizes (*n*) for each treatment are shown in (a). Asterisks indicate post hoc pairwise comparisons: * $p < 0.05$, and ** $p < 0.025$.

2010). We also observed that female pupfish maintained at 34 °C exhibited lower relative levels of mRNAs encoding steroidogenic enzymes *cyp19a1a*, *3βhsd*, and *hsd17β3* in the ovary. Ovarian aromatase mediates the synthesis of E_2 from T (Guigen et al., 2010; Young et al., 2005), and the lower abundance of *cyp19a1a* transcripts in females at 34 °C suggests reduced gonadal E_2 production at higher temperatures. Similarly, if the lower ovarian abundance of *3βhsd* and *hsd17β3* mRNAs in females maintained at 34 °C is indicative of reduced activity for these steroidogenic enzymes, then ovarian synthesis of 17α-hydroxyprogesterone and androstenedione, as well as the rate of androstenedione conversion to T, might be lower in females at 34 °C than at 24 °C, ultimately providing less T for E_2 synthesis (Young et al., 2005). Supporting this idea, a recent study in the related *Cyprinodon variegatus* pupfish revealed that fish maintained at 37 °C showed a similar pattern of altered mRNA abundances for gonadal *fshr*, *lhcgr*, *3βhsd, hsd17β3,* and *cyp19a1a* that was accompanied by lower ovarian GSI, a reduction in spawning capable oocytes, and lower circulating E_2 (Bock et al., 2021), implying that the ovarian mRNA changes observed here in *C. n. amargosae* may also result in altered ovarian gametogenesis. What is more, reductions in ovarian *cyp19a1a* have now been observed in several fishes exposed to high temperatures including Atlantic salmon (*Salmo salar*, Watts et al., 2004; Anderson et al., 2012a), coho salmon (*Oncorhynchus kisutch,* Anderson et al., 2019), and pejerrey (*Odontesthes bonariensis*, Elisio et al., 2012), implying that reduced E_2 synthesis is likely a key mechanism underlying reduced reproductive performance of female fish at elevated temperatures (reviewed by Alix et al., 2020; Servili et al., 2020).

The low volume of blood obtainable from pupfish prevented us from

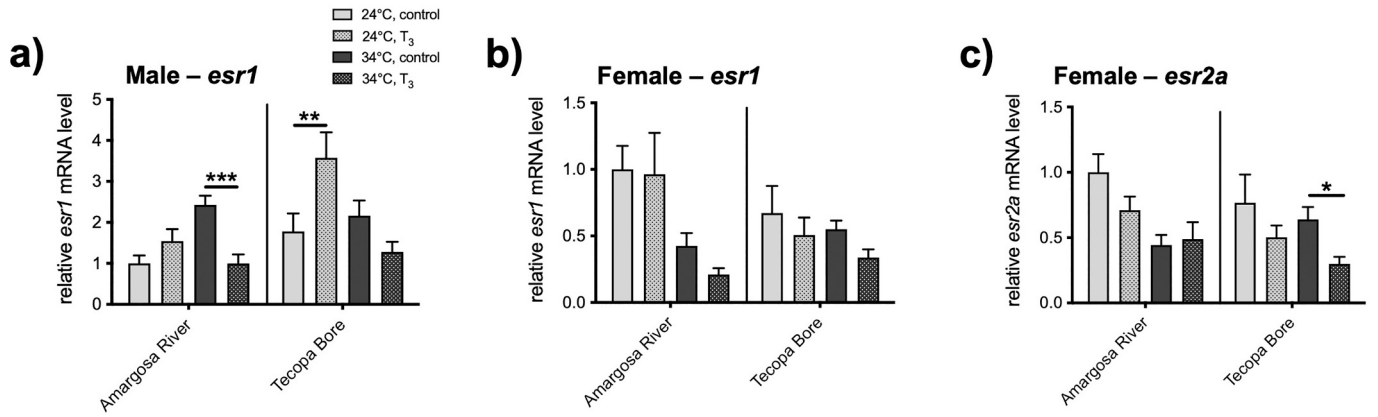


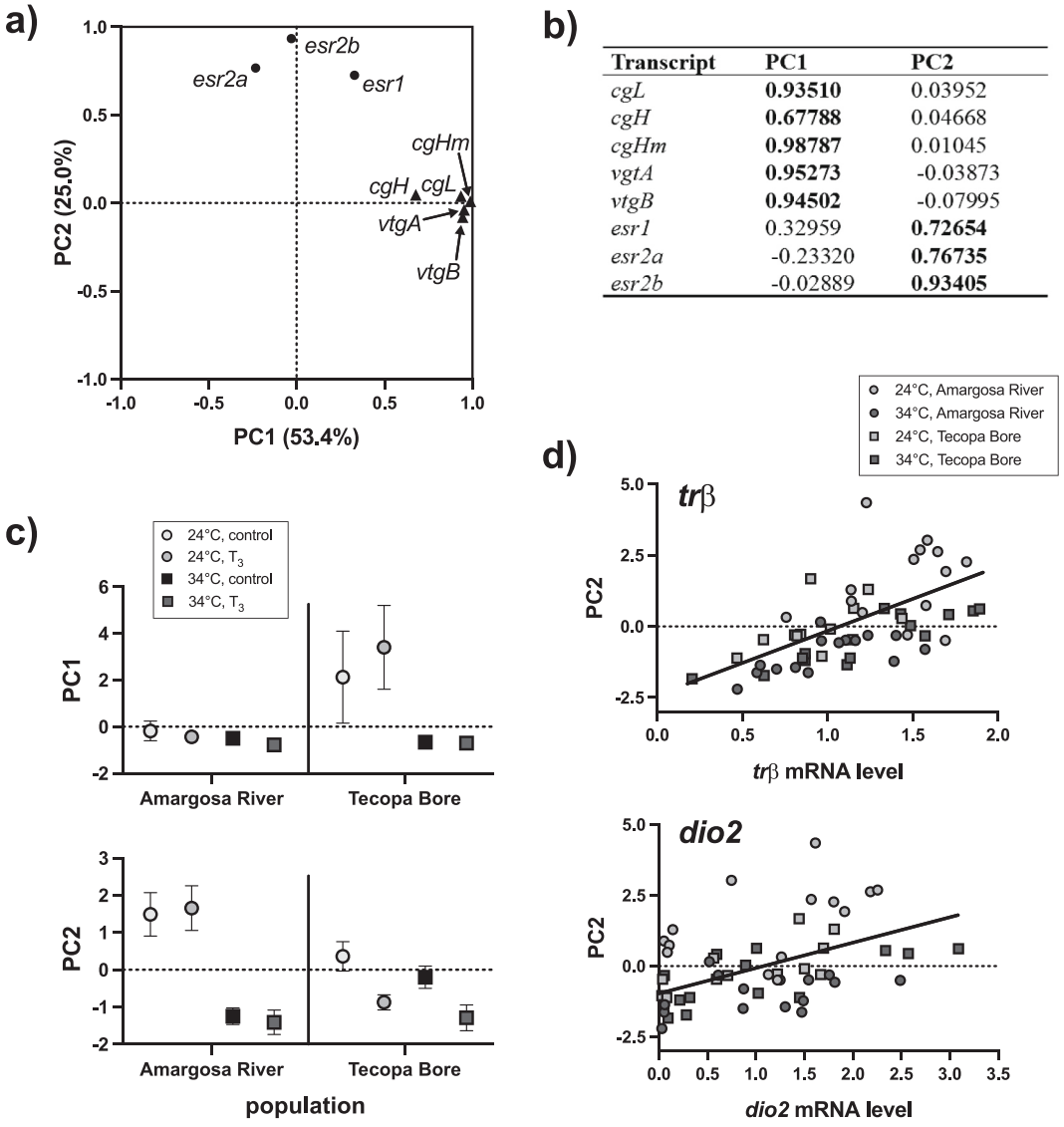
Fig. 7. T_3 and temperature interactions on liver *esr1* estrogen receptor mRNA levels in (a) male and (b) female pupfish, and (c) liver *esr2a* mRNA levels in females only. Sample sizes (*n*) are 7–10 fish per group, with * $p < 0.05$, ** $p < 0.025$, and *** $p < 0.0001$.

quantifying E_2 concentrations in addition to T_3. Nonetheless, if ovarian E_2 production was indeed reduced in pupfish maintained at 34 °C compared to fish at 24 °C, that difference might be evident in the expression patterns of estrogen-responsive genes. Along those lines, we observed that the abundances of mRNAs encoding the egg yolk vitellogenins and egg envelope choriogenins were significantly lower in the liver of female pupfish from Tecopa Bore at 34 °C than at 24 °C. It is well established in fish that E_2 promotes the synthesis and release of egg yolk precursor vitellogenin proteins (Bowman et al., 2000; Folmar et al., 2000; Denslow et al., 2001a, 2001b; Johnson and Lema, 2017), as well as choriogenins that will form the egg envelope of the oocyte (Arukwe and Goksoyr, 2003; Denslow et al., 2001a, 2001b; Johnson and Lema, 2017). Here, we observed that both female and male pupfish at 34 °C exhibited a lower abundance of transcripts encoding estrogen receptors *esr1* and *esr2b*, two of the three nuclear estrogen receptors commonly present in fishes (Hawkins et al., 2000). In fish, these nuclear receptors are autoinduced by estrogens (Yadetie et al., 1999; Arukwe et al., 2001). Lower relative mRNA levels of hepatic Vtg and Cg genes in female pupfish at 34 °C – coupled with lower estrogen receptor mRNA levels at this same temperature – is consistent with high temperatures having inhibited ovarian E_2 production in female pupfish to, ultimately, result in a downregulation or impairment of oogenesis. That interpretation would again be consistent with effects observed recently in *Cyprinodon variegatus*, where exposure of females to 37 °C led to lower circulating E_2 and corresponding reductions in both ovarian *cyp19a1a* mRNA levels and liver vitellogenin and choriogenin gene expression (Bock et al., 2021). Such reductions in liver vitellogenin or choriogenin production associated with elevated temperatures have similarly been observed in other fishes (King et al., 2003; Pankhurst et al., 2011; Pérez et al., 2011; Anderson et al., 2012b).

While our results here provide evidence for temperature-associated changes in HPG endocrine signaling in *C. n. amargosae*, the magnitude of those changes varied considerable between the Amargosa River and Tecopa Bore populations, suggesting that these two populations – while isolated for <55 years – exhibit differential sensitivities of reproduction to elevated temperatures. Thermal tolerance ranges have been documented to vary among pupfish taxa (e.g., Feldmeth, 1981; Beitinger et al., 2000; Nordlie, 2006), including allopatric populations of *C. nevadensis* pupfishes that have been isolated for only a few thousand years (Hirshfield et al., 1980). Local adaptation of thermal tolerance has thus evolved in pupfish experiencing differing temperature regimes. Our finding that the Amargosa River and Tecopa Bore pupfish populations vary in temperature effects on several key reproductive gene pathways including ovarian *cyp19a1a* and liver vitellogenin and choriogenin expression may thus reflect contemporary population divergence in thermal scope. Future studies, however, are needed to unravel how those endocrine-associated gene expression differences between the Amargosa River and Tecopa Bore populations are attributable to genetic differences, epigenetic variation, and plastic acclimation effects (Lema, 2014; Lema and Kitano, 2013; see also Veilleux et al., 2018).

It has already been shown that the critical thermal ranges of *Cyprinodon* pupfishes are plastic and sensitive to several interacting factors including acclimation temperature (Lowe and Heath, 1969; Feldmeth et al., 1974; Feldmeth, 1981), the thermal stability of the environment (Brown and Feldmeth, 1971; Feldmeth et al., 1974; Feldmeth, 1981), and co-occurring physical conditions such as salinity (Otto and Gerking, 1973; Beitinger et al., 2000). And yet, understanding the ability of pupfish and other fishes to rapidly evolve differences in temperature ranges for reproduction *and* the role of plastic developmental or multigenerational acclimation of reproduction to temperature remains a critical gap in understanding of how fish populations will respond to warming conditions under a changing global climate. Recent studies into temperature effects on reproductive performance in the tropical marine damselfish *Acanthochromis polyacanthus* found that the temperature that a fish experiences during early life influences its offsprings' reproductive performance at differing temperatures (Donelson et al., 2014; Veilleux et al., 2018). In one study by Donelson et al. (2014), for instance, offspring of damselfish reared at higher temperatures were observed to have enhanced reproductive performance at elevated temperatures. While we are unaware of any similar multi-generational study on reproduction in *Cyprinodon* pupfish, Salinas and Munch (2012) did observe transgenerational effects of temperature on growth rate in *C. variegatus* pupfish, indicating that temperature experience may indeed have cross-generational sway on pupfish physiology.

Pupfish in Tecopa Bore routinely experience temperatures exceeding 36 °C, while temperature conditions in the Amargosa River fluctuate widely (up to 25 °C diurnally) and typically cool below 24 °C at night even in the hot summer months (Lema et al., 2019; Lema, unpub. data). Since our present study was conducted with pupfish collected as adults out of their habitats, the dissimilar thermal experience of fish used in this experiment could also help explain the observed differences in thermal effects on ovarian and hepatic mRNA expression between Tecopa Bore and Amargosa River fish. It is possible that Tecopa Bore females in this experiment exhibited heightened *cyp19a1a* expression at low temperatures as part of a thermal 'release' wherein females in the lower experimental temperature (24 °C) – which is lower than they would experience in Tecopa Bore – strongly upregulated steroidogenesis. The dramatic increase in liver vitellogenin and choriogenin expression observed in those Tecopa Bore females at 24 °C supports that possibility. Prior studies have suggested that, even though the majority of spawning activity in variable temperature habitats can occur under high temperatures in late afternoon (Lema et al., 2010), ovulation in



Transcript	PC1	PC2
cgL	**0.93510**	0.03952
cgH	**0.67788**	0.04668
cgHm	**0.98787**	0.01045
vgtA	**0.95273**	-0.03873
vtgB	**0.94502**	-0.07995
esr1	0.32959	**0.72654**
esr2a	-0.23320	**0.76735**
esr2b	-0.02889	**0.93405**

Fig. 8. (a) Principal Components Analysis (PCA) of liver relative mRNA abundance in female pupfish. PCA for the eight transcripts quantified in liver reduced into two independent PCs, which together explained 78.4% of observed variation. (b) Factor loadings for the PCA performed on liver mRNA abundance showed PC1 represented choriogenin and vitellogenin mRNA levels, while PC2 represented estrogen receptor gene transcript abundance variation. (c) Mean values of each PC1 were elevated at the lower 24 °C temperature in Tecopa Bore pupfish only, but not in Amargosa River pupfish, and were unaffected by T_3. Liver PC2 values were lower at the higher 34 °C temperature in the females from both populations, and were also reduced in T_3-treated Tecopa Bore females, but not in Amargosa River females. (d) Individual variation in liver PC2 in females showed positive relationships with liver gene expression of both thyroid hormone receptor β (*trβ*) and type 2 iodothyronine deiodinase (*dio2*). Those relationships did not vary between populations and or with temperature condition.

pupfishes primarily occurs at night when temperatures are lower (Echelle et al., 1973). Echelle et al. (1973) suggested that, during cooler temperatures at night, pupfish may undergo a "critical repair period" (Lowe and Heath, 1969) that involves shifts in energy metabolism and increased ovarian activity (Echelle et al., 1973). Yet for pupfish living at consistently high temperatures – including pupfish in Tecopa Bore – such a period of recovery may not occur as temperatures may not decline sufficiently at night. Instead, Tecopa Bore pupfish may either be moving into microhabitat areas with sufficiently low temperatures to support oogenesis, or might have genetic evolutionary or epigenetic regulatory differences that enable reproduction at consistently high temperatures. In either case, additional studies are need to explore those questions further.

4.2. T_3 modulation of estrogen receptors and StAR

The data presented here suggest that TH modulation of reproductive pathways can occur via TH alterations to liver estrogen receptor expression as well as gonadal *star* mRNA levels. Interestingly, we observed that T_3 downregulated liver estrogen receptor expression in females from the Tecopa Bore population, but not from the Amargosa River population. Although few studies to date have examined TH effects on estrogen receptor expression in fish, Nelson and Habibi (2016) observed that goldfish given an intraperitoneal injection of T_3 responded by upregulating *esr1* mRNA abundance and downregulating *esr2a* mRNAs in the liver, while showing no changes in *esr2b* transcript abundance in that same tissue. Using culture hepatocytes from goldfish, Nelson and Habibi (2016) also showed that T_3 upregulation of *esr1* was dependent on the expression of TH receptors (goldfish *trα1* and *trβ*), and that these effects of T_3 on liver estrogen receptor expression function in

part to prime the liver for E_2-mediated upregulation of Vtg. Although the current experiment here with pupfish was not designed to test that same mechanism of TH potentiation of liver sensitivity to E_2, our findings offer further support for T_3 modulation of liver estrogen receptor expression in female fish, albeit in population-specific patterns. Those population-specific patterns might be explained in part by the observed positive correlation between individual liver PC2 values, which represent estrogen receptor mRNA expressional variation, and liver *trβ* mRNA levels. Lema et al. (2016) found that Amargosa River pupfish show elevated liver *trβ* mRNA expression at 24 °C compared to 34 °C, while Tecopa Bore fish show the opposing pattern with higher liver *trβ* mRNA levels at 34 °C. If those population differences in *trβ* gene regulation equate to differences in liver TRβ protein expression, higher PC2 values in Amargosa River pupfish at 24 °C might be in part related to the positive relationship between liver *trβ* mRNAs and PC2 detected here (see, Fig. 8d). In any case, it is clear from the divergent patterns of T_3 effects on liver PC2 values between Amargosa River and Tecopa Bore pupfish that several interacting factors including environmental temperature and TH action can influence liver estrogen receptor gene expression in female pupfish. While we cannot fully explain the evolved genetic or plastic epigenetic mechanistic underpinnings of those population differences at this time, the observed population variation in T_3 modulation of liver estrogen receptor expression supports the idea that TH effects on reproductive endocrine pathways can show complex patterns of seasonal-, sex-, and population-specific variation (e.g., Ma et al., 2020a).

Variation in gonadotropin-induced stimulation of steroidogenesis in ovarian tissues is regulated indirectly via changes in the expression of StAR (Nunez and Evans, 2007), which transports cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where it is converted to pregnenolone by P450scc (Stocco et al., 2005). Both female and male pupfish treated with T_3 had higher relative gonad abundances of *star* mRNAs compared to pupfish not treated with hormone. Short term (8 h) increases in T_3 have been demonstrated previously to induce *star* mRNA and StAR protein expression – as well as steroid production – in mammalian Leydig cells (Manna et al., 1999, 2001a, 2001b). In mammals, THs regulate testicular Sertoli cell differentiation and steroidogenesis by Leydig cells (Gao et al., 2014; Palmero et al., 1995). In fish, TH receptor expression likewise is present in the gonads (e.g., Lema et al., 2009), and THs have been found to regulate testicular Sertoli cell number (Matta et al., 2002), as well as T production (Jacob et al., 2005). THs have also been observed to enhance gonadotropin-mediated E_2 production from the fish ovary (Cyr and Eales, 1988, 1996; see also Blanton and Specker, 2007). Our observation of T_3-induced elevations in *star* mRNAs in both the testicular and ovarian tissues of pupfish suggests that THs may be able to affect gonadal function by altering mitochondrial cholesterol uptake either via T_3-induction of StAR expression directly or, possibly, through a more general effect of THs on mitochondrial biogenesis (Lombardi et al., 2015; Weitzel and Iwen, 2011), given that T_3 induction of mitochondrial biogenesis occurs via TH receptor-dependent regulation of the mitogenome in mammals (Weitzel et al., 2003). Since pupfish from both the 24 °C and 34 °C conditions showed similar T_3-induction of gonadal *star* mRNA levels ($p \geq 0.367$) – and given that these findings agree with previous observations in mammals (Manna et al., 1999, 2001a, 2001b) – we consider it likely that these T_3 effects will be reproducible in other fishes. Nonetheless, since plasma T_3 concentrations in the waterborne T_3-treated pupfish from this experiment exceeded normal physiological range (for additional details, see Lema et al., 2016), our observation that T_3 induces gonadal *star* transcription should be explored further in future experiments.

4.3. Conclusions

The data collected in this study indicate that female pupfish showed greater sensitivity to temperature-induced alterations of reproductive gene expression than males (see also, Bock et al., 2021). An overview of how temperature affected gene pathways involved in steroidogenesis and oogenesis in pupfish females is provided in Fig. 9. The picture that emerges from those gene expression responses points to females from the abidingly warm habitat of Tecopa Bore experiencing larger temperature-mediated shifts in E_2 steroidogenesis compared to

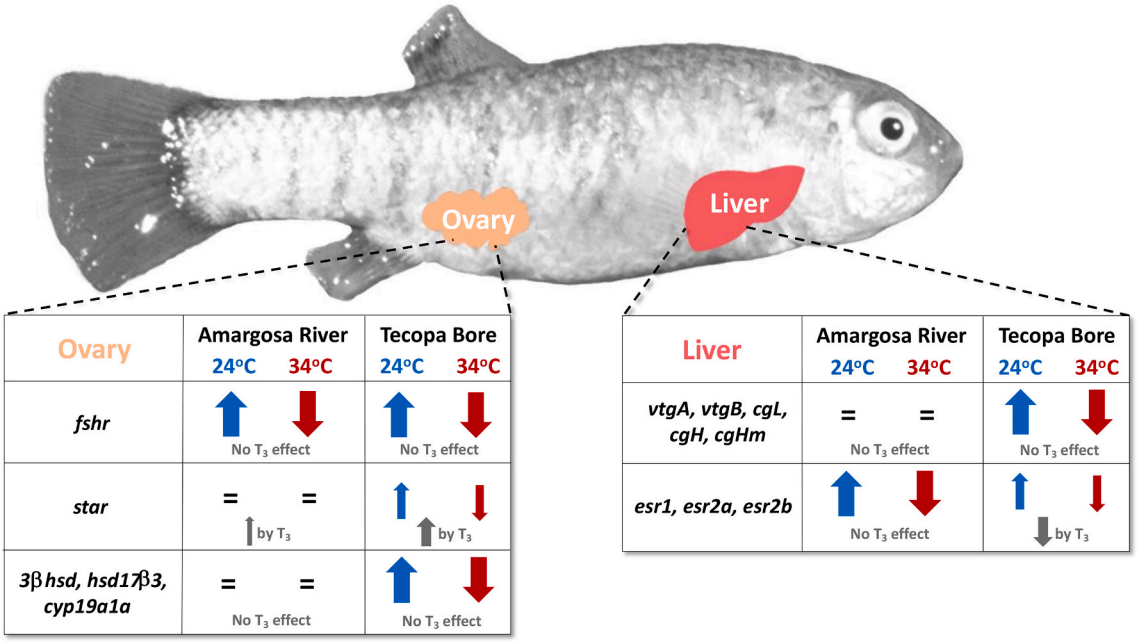


Fig. 9. Summary of temperature and T_3 effects on ovarian and hepatic reproductive gene expression in female *C. n. amargosae* pupfish from the allopatric Amargosa River and Tecopa Bore populations. Arrows denote higher or lower relative mRNA levels of each gene transcript in a given population exposed to conditions of 24 °C (*blue arrows*) or 34 °C (*red arrows*). Equal sign symbols indicate 'no difference' in relative expression level of that transcript between temperatures in that population. T_3 effects on each transcript are indicated in gray text below each set of temperature response arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Amargosa River females, as suggested by the consistent pattern of larger changes in ovarian *fshr, star,* and steroidogenic enzyme gene expression. Those changes in ovarian gene expression were paralleled by greater temperature-related modifications to liver vitellogenin, choriogenin, and estrogen receptor gene expression in females from Tecopa Bore compared to the Amargosa River population, implying that Tecopa Bore females experienced greater alterations to E_2 induction of liver oogenesis gene expression at the different temperatures. Interestingly, T_3 effects on ovarian *star* and liver estrogen receptor gene expression were also more pronounced in Tecopa Bore females. Prior work revealed that T_3 upregulated relative levels of liver *dio3* mRNAs in pupfish from both the Amargosa River and Tecopa Bore populations at 34 °C, but not at 24 °C (Lema et al., 2016). That same study also observed population differences in liver *trβ* expression responses to temperature – suggesting that nearer-term (in this case, 88 d) thermal experience may shape E_2 induction of liver vitellogenesis and choriogenesis in part by modulating local TH deiodination dynamics (i.e., T_3 availability) and TH receptor expression (i.e., tissue sensitivity to TH action) in population-specific patterns, with consequence for T_3 regulation of liver *esr1* and *esr2a* expression. Whether endocrine dissimilarities between females from the Amargosa River and Tecopa Bore populations originated from longer-term developmental influences (i.e., early life experience), transgenerational effects, or evolved population-level variation is not clear. Even so, observed differences between these populations in how T_3 modulated liver *esr1* and *esr2a* expression make clear that even closely-related fish populations can differ in temperature regulation of reproduction, and point to a role for TH signaling as a contributing mechanism underlying population variation in temperature regulation of gonadal steroidogenesis and oogenesis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by a California State University (CSU) Program for Education and Research in Biotechnology (CSUPERB) Development Grant to S.C.L., and by Cal Poly College Based Fees (CBF) funding to M.I.C. Pupfish were collected under permission of the California Department of Fish and Wildlife (Scientific Collecting Permit #4793). The authors thank Rob Brewster for assistance with construction of the animal holding facilities, and two anonymous reviewers for insightful critique and suggestions that improved the quality of this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2022.111280.

References

Alix, M., Kjesbu, O.S., Anderson, K.C., 2020. From gametogenesis to spawning: how climate-driven warming affects teleost reproductive biology. J. Fish Biol. 97, 607–632.

Allan, E.R.O., Habibi, H.R., 2012. Direct effects of triiodothyroinine on production of anterior pituitary hormones and gonadal steroids in goldfish. Mol. Reprod. Dev. 79, 592–602.

Anderson, K., Swanson, P., Pankhurst, N., King, H., Elizur, A., 2012a. Effect of thermal challenge on plasma gonadotropin levels and ovarian steroidogenesis in female maiden and repeat spawning Tasmanian Atlantic salmon (*Salmo salar*). Aquaculture 334-337, 205–212.

Anderson, K., King, H., Pankhurst, N., Ruff, N., Pankhurst, P., Elizur, A., 2012b. Effect of elevated temperature on estrogenic induction of vitellogenesis and zonagenesis in juvenile Atlantic salmon (*Salmo salar*). Mar. Freshw. Behav. Physiol. 45, 1–15.

Anderson, K., Luckenbach, J.A., Yamamoto, Y., Elizur, A., 2019. Impacts of Fsh, Igf1, and high temperature on the expression of genes involved in steroidogenesis, cell communication, and apoptosis in isolated coho salmon previtellogenic ovarian follicles. Aquaculture 506, 60–69.

Arukwe, A., Goksoyr, A., 2003. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. Comp. Hepatol. 2, 4.

Arukwe, A., Kullman, S.W., Hinton, D.E., 2001. Differential biomarker gene and protein expressions in nonylphenol and estradiol-17β treated juvenile rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. 129, 1–10.

Barlow, G.W., 1961. Social behavior of the desert pupfish, *Cyprinodon macularius*, in the field and in the aquarium. Am. Midl. Nat. 65, 339–359.

Beitinger, T.L., Bennett, W.A., McCauley, R.W., 2000. Temperature tolerances of north American freshwater fishes exposed to dynamic changes in temperature. Environ. Biol. Fish 58, 237–275.

Bennett, W.A., Beitinger, T.L., 1997. Temperature tolerance of the sheepshead minnow, *Cyprinodon variegatus*. Copeia 1997, 77–87.

Blanton, M.L., Specker, J.L., 2007. The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. Crit. Rev. Toxicol. 37, 97–115.

Blewett, T.A., Robertson, L.M., MacLatchy, D.L., Wood, C.M., 2013a. Impact of environmental oxygen, exercise, salinity, and metabolic rate on the uptake and tissue-specific distribution of 17α-ethynylestradiol in the euryhaline teleost *Fundulus heteroclitus*. Aquat. Toxciol. 138-139, 43–51.

Blewett, T.A., MacLatchy, D.L., Wood, C.M., 2013b. The effects of temperature and salinity on 17α-ethynylestradiol uptake and its relationship to oxygen consumption in the model euryhaline teleost (*Fundulus heteroclitus*). Aquat. Toxicol. 127, 61–71.

Bock, S.L., Chow, M.I., Forsgren, K.L., Lema, S.C., 2021. Widespread alterations to hypothalamic-pituitary-gonadal (HPG) signaling underlie high temperature reproductive inhibition in the eurythermal sheepshead minnow (*Cyprinodon variegatus*). Mol. Cell. Endocrinol. 537, 111447.

Bowman, C.J., Kroll, K.J., Hemmer, M.J., Folmar, L.C., Denslow, N.D., 2000. Estrogen-induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). Gen. Comp. Endocrinol. 120, 300–313.

Brown, J.H., Feldmeth, C.R., 1971. Evolution in constant and fluctuating environments: thermal tolerances of desert pupfish (*Cyprinodon*). Evolution 25, 390–398.

Carson, E.W., De la Maza-Benignos, M., Lozano-Vilano, M., Vela-Valladares, L., Banda-Villanueva, I., Turner, T.F., 2014. Conservation genetic assessment of the critically endangered Julimes pupfish, *Cyprinodon julimes*. Conserv. Genet. 15, 483–488.

Castañeda Cortés, D.C., Langlois, V.S., Fernandino, J.I., 2014. Crossover of the hypothalamic pituitary–adrenal/interrenal, –thyroid, and –gonadal axes in testicular development. Front. Endocrinol. 5, 139.

Chown, S.L., Hoffmann, A.A., Kristensen, T.N., Angilletta Jr., M.J., Stenseth, N.C., Pertoldi, C., 2010. Adapting to climate change: a perspective from evolutionary physiology. Clim. Res. 43, 3–15.

Cyr, D.G., Eales, J.G., 1988. *In vitro* effects of thyroid hormones on gonadotropin-induced estradiol-17β secretion by ovarian follicles of rainbow trout, *Salmo gairdneri*. Gen. Comp. Endocrinol. 69, 80–87.

Cyr, D.G., Eales, J.G., 1996. Interrelationships between thyroidal and reproductive endocrine systems in fish. Rev. Fish Biol. Fish. 6, 165–200.

Deal, C.K., Volkoff, H., 2020. The role of the thyroid axis in fish. Front. Endocrinol. 11, 596585.

Denslow, N.D., Bowman, C.J., Ferguson, R.J., Lee, H.S., Hemmer, M.J., Folmar, L.C., 2001a. Induction of gene expression in sheepshead minnows (*Cyprinodon variegatus*) treated with 17β-estradiol, diethylstilbestrol, or ethinylestradiol: the use of mRNA fingerprints as an indicator of gene regulation. Gen. Comp. Endocrinol. 121, 250–260.

Denslow, N.D., Lee, H.S., Bowman, C.J., Hemmer, M.J., Folmar, L.C., 2001b. Multiple responses in gene expression in fish treated with estrogen. Comp. Biochem. Physiol. B 129, 277–282.

Dickhoff, W.W., Folmar, L.C., Mighell, J.L., Mahnken, C.V., 1982. Plasma thyroid hormones during smoltification of yearling and underyearling coho salmon and yearling Chinook salmon and steelhead trout. Aquaculture 28, 39–48.

Dittrich, R., Beckmann, M.W., Oppelt, P.G., Hoffmann, I., Lotz, L., Kuwert, T., Mueller, A., 2011. Thyroid hormone receptors and reproduction. J. Reprod. Immunol. 90, 58–66.

Donelson, J.M., Munday, P.L., McCormick, M.I., Pankhurst, N.W., Pankhurst, P.M., 2010. Effects of elevated water temperature and food availability on the reproductive performance of a coral reef fish. Mar. Ecol. Prog. Ser. 401, 233–243.

Donelson, J.M., McCormick, M.I., Booth, D.J., Munday, P.L., 2014. Reproductive acclimation to increased water temperature in a tropical reef fish. PLoS One 9, e97223.

Dorts, J., Grenouillet, G., Douxfils, J., Mandiki, S.N.M., Milla, S., Silvestre, F., Kestemont, P., 2012. Evidence that elevated water temperature affects the reproductive physiology of the European bullhead *Cottus gobio*. Fish Physiol. Biochem. 38, 389–399.

Duarte-Guterman, P., Navarro-Martín, L., Trudeau, V.L., 2014. Mechanisms of crosstalk between endocrine systems: regulation of sex steroid hormone synthesis and action by thyroid hormones. Gen. Comp. Endocrinol. 203, 69–85.

Eales, J.G., Chang, J.P., van der Kraak, G., Omeijaniuk, R.J., Uin, L., 1982. Effects of temperature on plasma thyronine and iodine kinetics in rainbow trout, *Salmo gairderi*. Gen. Comp. Endocrinol. 47, 295–307.

Echelle, A.A., 1973. Behavior of the pupfish, *Cyprinodon rubrofluviatilis*. Copeia 1973, 68–76.

Echelle, A.A., Wilson, S., Hill, L.G., 1973. The effects of four temperature-daylength combinations on ovogenesis in the Red River pupfish, *Cyprinodon rubrofluviatilis* (Cyprinodontidae). Southwest. Nat. 18, 229–239.

Elisio, M., Chalde, T., Miranda, L.A., 2012. Effects of short periods of warm water fluctuations on reproductive endocrine axis of the pejerrey (*Odontesthes bonariensis*) spawning. Comp. Biochem. Physiol. A 163, 47–55.
Feldmeth, C.R., 1981. The evolution of thermal tolerance in desert pupfish (genus *Cyprinodon*). In: Naiman, J., Soltz, D.L. (Eds.), Fishes of North American Deserts. John Wiley and Sons, New York, NY, pp. 357–384.
Feldmeth, C.R., Stone, E.A., Brown, J.H., 1974. An increased scope for thermal tolerance upon acclimating pupfish (*Cyprinodon*) to cycling temperatures. J. Comp. Physiol. 89, 39–44.
Feng, K., Zhu, K., Wu, Z., Su, S., Yao, W., 2022. Identification and expression analysis of thyroid-stimulating hormone β subunit, and effects of T3 on gonadal differentiation-related gene expression in rice field eel, *Monopterus albus*. Comp. Biochem. Physiol. B 258, 110681.
Feuerbacher, O.G., Mapula, J.A., Bonar, S.A., 2015. Propagation of hybrid devils hole pupfish x ash meadows Amargosa pupfish. N. Am. J. Aquacult. 77, 513–523.
Flood, D.E., Fernandino, J.I., Langlois, V.S., 2013. Thyroid hormones in male reproductive development: evidence for direct crosstalk between the androgen and thyroid hormone axes. Gen. Comp. Endocrinol. 192, 2–14.
Folmar, L.C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., Denslow, N.D., 2000. Compartive estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (*Cyprinodon variegatus*), vitellogenin bioassay. Aquat. Toxicol. 49, 77–88.
Gao, Y., Lee, W.M., Cheng, Y., 2014. Thyroid hormone function in the rat testis. Front. Endocrinol. 5, 188.
Gerking, S.D., Lee, R.M., 1983. Thermal limits for growth and reproduction in the desert pupfish *Cyprinodon n. nevadensis*. Physiol. Zool. 56, 1–9.
Gerking, S.D., Lee, R., Shrode, J.B., 1979. Effects of generation-long temperature acclimation on reproductive performance of the desert pupfish, *Cyprinodon n. nevadensis*. Physiol. Zool. 52, 113–121.
Gillet, C., Breton, B., 2009. LH secretion and ovulation following exposure of Arctic charr to different temperature and photoperiod regimes: responsiveness of females to a gonadotropin-releasing hormone analogue and a dopamine antagonist. Gen. Comp. Endocrinol. 162, 210–218.
Glasser, F., Mikolajczyk, T., Jalabert, B., Baroiller, J.-F., Breton, B., 2004. Temperature effects along the reproductive axis during spawning induction of grass carp (*Ctenopharyngodon idella*). Gen. Comp. Endocrinol. 136, 171–179.
Guigen, Y., Fostier, A., Piferrer, F., Chang, C.-F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165, 352–366.
Habibi, H.R., Nelson, E.R., Allan, E.R., 2012. New insights into thyroid hormone function and modulation of reproduction in goldfish. Gen. Comp. Endocrinol. 175, 19–26.
Hani, Y.M.I., Turies, C., Palluel, O., Delahaut, L., Bado-Nilles, A., Geffard, A., Dedourge-Geffard, O., Porcher, J.M., 2019. Effects of a chronic exposure to different water temperatures and/or to an environmental cadmium concentration on the reproduction of the threespine stickleback (*Gasterosteus aculeatus*). Ecotoxicol. Environ. Saf. 174, 48–57.
Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., Thomas, P., 2000. Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc. Natl. Acad. Sci. U. S. A. 97, 10751–10756.
Heath, A.G., Turner, B.J., Davis, W.P., 1993. Temperature preference and tolerances of three fish species inhabiting hyperthermal ponds on mangrove islands. Hydrobiologia 259, 47–55.
Hirshfield, M.F., Feldmeth, C.R., Soltz, D.L., 1980. Genetic differences in physiological tolerances of Amargosa pupfish (*Cyprinodon nevadensis*) populations. Science 207, 999–1001.
Hoffman, A.A., Sgrò, C.M., 2011. Climate change and evolutionary adaptation. Nature 470, 479–485.
Huynh, T.B., Fairgrieve, W.T., Hayman, E.S., Lee, J.S., Luckenbach, J.A., 2019. Inhibition of ovarian development and instances of sex reversal in genotypic female sablefish (*Anoplopoma fimbria*) exposed to elevated water temperature. Gen. Comp. Endocrinol. 279, 88–98.
Jacob, T.N., Pandey, J.P., Raghuveer, K., Sreenivasulu, G., Gupta, A.D., Yoshikuni, M., Jagota, A., Senthilkumaran, B., 2005. Thyroxine-induced alterations in the testis and seminal vesicles of air-breathing catfish, *Claria gariepinus*. Fish Physiol. Biochem. 31, 271–274.
Jannini, E.A., Ulisse, S., D'Armiento, M., 1995. Thyroid hormone and male gonadal function. Endocr. Rev. 16, 443–459.
Johnson, K.M., Lema, S.C., 2017. Temporal patterns of induction and recovery of biomarker transcriptional responses to 4-nonylphenol and 17β-estradiol in the estuarine arrow goby, *Clevelandia ios*. Environ. Toxicol. 32, 1513–1529.
Johnston, C.E., Eales, J.G., 1995. Effects of acclimation and assay temperature on outer-ring and inner-ring thyroxine and 3,5,3′-triiodo-L-thryonine deiodination by liver-microsomes of rainbow trout, *Oncorhynchus mykiss*. J. Exp. Zool. 272, 426–434.
King, H.R., Pankhurst, N.W., 2004. Effect of maintenance at elevated temperatures on ovulation and luteinizing hormone releasing hormone analogue responsiveness of female Atlantic salmon (*Salmo salar*) in Tasmania. Aquaculture 233, 583–597.
King, H.R., Pankhurst, N.W., Watts, M., Pankhurst, P.M., 2003. Effect of elevated summer temperatures on gonadal steroid production, vitellogenesis and egg quality in female Atlantic salmon. J. Fish Biol. 62, 153–167.
King, H.R., Pankhurst, N.W., Watts, M., 2007. Reproductive sensitivity to elevated water temperatures in female Atlantic salmon is heightened at certain stages of vitellogenesis. J. Fish Biol. 70, 190–205.
Kitano, T., Takamune, K., Kobayashi, T., Nagahama, Y., Abe, S.-I., 1999. Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*). J. Mol. Endocrinol. 23, 167–176.
Kusakabe, M., Nakamura, I., Evans, J., Swanson, P., Young, G., 2006. Changes in mRNAs encoding steroidogenic acute regulatory protein, steroidogenic enzymes and receptors for gonadotropins during spermatogenesis in rainbow trout testes. J. Endocrinol. 189, 541–554.
Lema, S.C., 2010. Identification of multiple vasotocin receptor cDNAs in teleost fish: sequences, phylogenetic analysis, sites of expression, and regulation in the hypothalamus and gill in response to hyperosmotic challenge. Mol. Cell. Endocrinol. 321, 215–230.
Lema, S.C., 2014. Hormones and phenotypic plasticity in an ecological context: linking physiological mechanisms to evolutionary processes. Integr. Comp. Biol. 54, 850–863.
Lema, S.C., 2020. Hormones, developmental plasticity, and adaptive evolution: endocrine flexibility as a catalyst for 'plasticity-first' phenotypic divergence. Mol. Cell. Endocrinol. 502, 110678.
Lema, S.C., Kitano, J., 2013. Hormones and phenotypic plasticity: implications for the evolution of integrated adaptive phenotypes. Curr. Zool. 59, 506–525.
Lema, S.C., Dickey, J.T., Schultz, I.R., Swanson, P., 2009. Thyroid hormone regulation of mRNAs encoding thyrotropin β-subunit, glycoprotein α-subunit, and thyroid hormone receptors α and β in brain, pituitary gland, liver, and gonads of an adult teleost, *Pimephales promelas*. J. Endocrinol. 202, 43–54.
Lema, S.C., Wagstaff, L.J., Gardner, N.M., 2010. Diurnal rhythms of behavior and brain mRNA expression for arginine vasotocin, isotocin, and their receptors in wild Amargosa pupfish (*Cyprinodon nevadensis amargosae*). Mar. Freshw. Behav. Physiol. 43, 257–281.
Lema, S.C., Sanders, K.E., Walti, K.A., 2015. Arginine vasotocin, isotocin and nonapeptide receptor gene expression link to social status and aggression in sex-dependent patterns. J. Neuroendocrinol. 27, 142–157.
Lema, S.C., Chow, M.I., Resner, E.J., Westman, A.A., May, D., Dittman, A.H., Hardy, K. M., 2016. Endocrine and metabolic impacts of warming aquatic habitats: differential responses between recently-isolated populations of a eurythermal desert pupfish. Conserv. Physiol. 6, cow047.
Lema, S.C., Bock, S.L., Malley, M.M., Elkins, E.A., 2019. Warming waters beget smaller fish: evidence for reduced size and altered morphology in a desert fish following anthropogenic temperature change. Biol. Lett. 15, 20190518.
Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412–437.
Little, A.G., Kunisue, T., Kannan, K., Seebacher, F., 2013. Thyroid hormone actions are temperature-specific and regulate thermal acclimation in zebrafish (*Danio rerio*). BMC Biol. 11, 1–15.
Liu, R.K., 1969. The Comparative Behavior of Allopatric Species (Teleostei-Cyprinodontidae: *Cyprinodon*). Dissertation. University of California, Los Angeles.
Lombardi, A., Moreno, M., de Lange, P., Iossa, S., Busiello, R.A., Goglia, F., 2015. Regulation of skeletal muscle mitochondrial activity by thyroid hormones: focus on the "old" triiodothyronine and the "emerging" 3,5-diiodothyronine. Front. Physiol. 6, 237.
Lowe, C.H., Heath, W.G., 1969. Behavioral and physiological responses to temperature in the desert pupfish *Cyprinodon macularius*. Physiol. Zool. 42, 53–59.
Ma, Y., Ladisa, C., Chang, J.P., Habibi, H.R., 2020a. Multifactorial control of reproductive and growth axis in male goldfish: influences of GnRH, GnIH and thyroid hormone. Mol. Cell. Endocrinol. 500, 110629.
Ma, Y., Ladisa, C., Chang, J.P., Habibi, H.R., 2020b. Seasonal related multifactorial control of pituitary gonadotropin and growth hormone in female goldfish: influences of neuropeptides and thyroid hormone. Front. Endocrinol. 11, 175.
Manna, P.R., Tena-Sempere, M., Huhtaniemi, I.T., 1999. Molecular mechanisms of thyroid hormone-stimulated steroidogenesis in mouse Leydig tumor cells: involvement of the steroidogenic acute regulatory (StAR) protein. J. Biol. Chem. 274, 5909–5918.
Manna, P.R., Kero, J., Tena-Sempere, M., Pakarinen, P., Stocco, D.M., Huhtaniemi, I.T., 2001a. Assessment of mechanisms of thyroid hormone action in mouse Leydig cells: regulation of the steroidogenic acute regulatory protein, steroidogenesis, and luteinizing hormone receptor function. Endocrinology 142, 319–331.
Manna, P.R., Roy, P., Clark, B.J., Stocco, D.M., Huhtaniemi, I.T., 2001b. Interaction of thyroid hormone and steroidogenic acute regulatory (StAR) protein in the regulation of murine Leydig cell steroidogenesis. J. Steroid Biochem. Mol. Biol. 76, 167–177.
Matta, S.L.P., Vilela, D.A.R., Godinho, H.P., França, L.R., 2002. The goitrogen 6-n-propyl-2-thiouracil (PTU) given during testis development increase Sertoli and germ cell numbers per cyst in fish: the tilapia (*Oreochromis niloticus*). Endocrinology 143, 970–978.
Mazzeo, I., Peñaranda, D.S., Gallego, V., Baloche, S., Nourizadeh-Lillabadi, R., Tveiten, H., Sylvie Dufour, J.F., Asturiano, F.-A. Weltzien, Pérez, L., 2014. Temperature modulates the progression of vitellogenesis in the European eel. Aquaculture 434, 38–47.
Minckley, W.L., Minckley, C.O., 1986. *Cyprinodon pachycephalus*, a new species of pupfish (Cyprinodontidae) from the Chihuahuan desert of northern Mexico. Copeia 1986, 184–192.
Miranda, L.A., Chalde, T., Elisio, M., Strüssmann, C.A., 2013. Effects of global warming on fish reproductive endocrine axis, with special emphasis in pejerrey *Odontesthes bonariensis*. Gen. Comp. Endocrinol. 192, 45–54.
Motani, R., Wainwright, P.C., 2015. How warm is too warm for the life cycle of actinopterygian fishes? Sci. Report. 5, 11597.
Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Develop. Growth Differ. 50, S195–S219.
Naiman, R.J., 1974. Bioenergetics of a Pupfish Population (*Cyprinodon*) and its Algal Food Supply in a Thermal Stream. Ph.D. Dissertation. Arizona State University.

Nakamura, I., Evans, J.C., Kusakabe, M., Nagahama, Y., Young, G., 2005. Changes in steroidogenic enzyme and steroidogenic acute regulatory protein messenger RNAs in ovarian follicles during ovarian development of rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 144, 224–231.
Nelson, E.R., Habibi, H.R., 2016. Thyroid hormone regulates vitellogenin by inducing estrogen receptor alpha in the goldfish liver. Mol. Cell. Endocrinol. 436, 259–267.
Nelson, E.R., Allen, E.R.O., Pang, F.Y., Habibi, H.R., 2010. Thyroid hormone and reproduction: regulation of estrogen receptors in goldfish gonads. Mol. Reprod. Dev. 77, 784–794.
Nordlie, F.G., 2006. Physicochemical environments and tolerances of cyprinodontoid fishes found in estuaries and salt marshes of eastern North America. Rev. Fish Biol. Fish. 16, 51–106.
Nunez, B.S., Evans, A.N., 2007. Hormonal regulation of the steroidogenic acute regulatory protein (StAR) in gonadal tissues of the Atlantic croaker (*Micropogonias undulatus*). Gen. Comp. Endocrinol. 150, 495–504.
Otto, R.G., Gerking, S.D., 1973. Heat tolerance of a Death Valley pupfish (genus *Cyprinodon*). Physiol. Zool. 46, 43–49.
Palmero, S., Prati, M., Bolla, F., Fugassa, E., 1995. Tri-iodothyronine directly affects rat Sertoli cell proliferation and differentiation. J. Endocrinol. 145, 355–362.
Pankhurst, N.W., 2011. The endocrinology of stress in fish: an environmental perspective. Gen. Comp. Endocrinol. 170, 265–272.
Pankhurst, N.W., King, H.R., 2010. Temperature and salmonid reproduction: implications for aquaculture. J. Fish Biol. 76, 69–85.
Pankhurst, N.W., Munday, P.L., 2011. Effects of climate change on fish reproduction and early life history stages. Mar. Freshw. Res. 62, 1015–1026.
Pankhurst, N.W., Porter, M.J.R., 2003. Cold and dark or warm and light: variations on the theme of environmental control of reproduction. Fish Physiol. Biochem. 28, 385–389.
Pankhurst, N.W., Thomas, P.M., 1998. Maintenance at elevated temperature delays the steroidogenic and ovulatory responsiveness of rainbow trout *Oncorhynchus mykiss* to luteinizing hormone releasing hormone analogue. Aquaculture 166, 163–177.
Pankhurst, N.W., King, H.R., Anderson, K., Elizur, A., Pankhurst, P.M., Ruff, N., 2011. Thermal impairment of reproduction is differentially expressed in maiden and repeat spawning Atlantic salmon. Aquaculture 316, 77–87.
Pérez, L., Peñaranda, D.S., Dufour, S., Baloche, S., Palstra, A.P., Van Den Thillart, G.E.E. J.M., Asturiano, J.F., 2011. Influence of temperature regime on endocrine parameters and vitellogenesis during experimental maturation of European eel (*Anguilla anguilla*) females. Gen. Comp. Endocrinol. 174, 51–59.
Planas, J.V., Swanson, P., 2008. Physiological function of gonadotropins in fish. In: Rocha, M.J., Arukwe, A., Kapoor, B.G. (Eds.), Fish Reproduction. CRC Press, Boca Raton, FL, pp. 37–66.
Raine, J.C., 2011. Thyroid hormone and reproduction in fishes. In: Norris, D.O., Lopez, K. H. (Eds.), Hormones and Reproduction of Vertebrates, vol. 1. Academic Press, London, UK, pp. 83–102.
Salinas, S., Munch, S.B., 2012. Thermal legacies: transgenerational effects of temperature on growth in a vertebrate. Ecol. Lett. 15, 159–163.
Seager, R., Vecchi, G.A., 2010. Greenhouse warming and the 21st century hydroclimate of southwestern North America. Proc. Natl. Acad. Sci. U. S. A. 107, 21277–21282.
Seager, R., Ting, M., Held, I., Kushnir, Y., Lu, J., Vecchi, G., Huang, H.-P., Harnik, N., Leetmaa, A., Lau, N.-C., et al., 2007. Model predictions of an imminent transition to a more arid climate in southwestern North America. Science 316, 1181–1184.
Servili, A., Canario, A.V.M., Mouchel, O., Muñoz-Cueto, J.A., 2020. Climate change impacts on fish reproduxtion are mediated at multiple levels of the brain-pituitary-gonad axis. Gen. Comp. Endocrinol. 291, 113439.
Sharma, P., Patiño, R., 2013. Regulation of gonadal sex ratios and pubertal development by the thyroid endocrine system in zebrafish (*Danio rerio*). Gen. Comp. Endocrinol. 184, 111–119.
Shrode, J.B., Gerking, S.D., 1977. Effects of constant and fluctuating temperatures on reproductive performance of a desert pupfish, *Cyprinodon n. nevadensis*. Physiol. Zool. 50, 1–10.
Soltz, D.L., 1974. Variation in Life History and Social Organization of some Populations of Nevada Pupfish, *Cyprinodon nevadensis*. Ph.D. Dissertation. University of California, Los Angeles.
Soltz, D.L., Naiman, R.J., 1978. The natural history of native fishes in the Death Valley system. Nat. Hist. Mus. Los Angeles County. Sci. Ser. 30, 1–76.
Soria, F.N., Strüssmann, C.A., Miranda, L.A., 2008. High water temperatures impair the reproductive ability of the pejerrey fish *Odontesthes bonariensis*: effects on the hypophyseal-gonadal axis. Physiol. Biochem. Zool. 81, 898–905.
Stocco, D.M., Wang, X., Jo, Y., Manna, P.R., 2005. Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: more complicated that we thought. Mol. Endocrinol. 19, 2647–2659.
Suquet, M., Normant, Y., Gaignon, J.L., Quéméner, L., Fauvel, C., 2005. Effect of water temperature on individual reproductive activity of pollack (*Pollachius pollachius*). Aquaculture 243, 113–120.
Tovo-Neto, A., da Silva Rodrigues, M., Habibi, H.R., Nóbrega, R.H., 2018. Thyroid hormone actions on male reproductive system of teleost fish. Gen. Comp. Endocrinol. 265, 230–236.
Tveiten, H., Johnsen, H.K., 2001. Thermal influences on temporal changes in plasma testosterone and oestradiol-17β concentrations during gonadal recrudescence in female common wolfish. J. Fish Biol. 59, 175–178.
Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2004. An aromatase inhibitor or high water temperature induce oocyte apoptosis and depletion of P450 aromatase activity in the gonads of genetic female zebrafish during sex-reversal. Comp. Biochem. Physiol. A 137, 11–20.
Van Der Kraak, G., Pankhurst, N.W., 1997. Temperature effects on the reproductive performance of fish. In: Wood, C.M., McDonald, D.G. (Eds.), Global Warming: Implications for Freshwater and Marine Fish. Cambridge Univ. Press, Cambridge, UK, pp. 159–176.
Veilleux, H.D., Donelson, J.M., Munday, P.L., 2018. Reproductive gene expression in a coral reef fish exposed to increasing temperature across generations. Conserv. Physiol. 6, cox077.
Vikingstad, E., Andersson, E., Hansen, T.J., Norberg, B., Mayer, I., Stefansson, S.O., Fjelldal, P.G., Taranger, G.L., 2016. Effects of temperature on the final stages of sexual maturation in Atlantic salmon (*Salmo salar* L.). Fish Physiol. Biochem. 42, 895–907.
Watts, M., Pankhurst, N.W., King, H.R., 2004. Maintenance of Atlantic salmon (*Salmo salar*) at elevated temperature inhibits cytochrome P450 aromatase activity in isolated ovarian follicles. Gen. Comp. Endocrinol. 135, 381–390.
Webb, M.A.H., Van Eenennaam, J.P., Doroshov, S.I., Moberg, G.P., 1999. Preliminary observations on the effects of holding temperature on reproductive performance of female white sturgeon, *Acipenser transmontanus* Richardson. Aquaculture 176, 315–329.
Weitzel, J.M., Iwen, K.A., 2011. Coordination of mitochondrial biogenesis by thyroid hormone. Mol. Cell. Endocrinol. 342, 1–7.
Weitzel, J.M., Iwen, K.A.H., Seitz, H.J., 2003. Regulation of mitochondrial biogenesis by thyroid hormone. Exp. Physiol. 88, 121–128.
Wylie, M.J., Setiawan, A.N., Irvine, G.W., Symonds, J.E., Elizur, A., Dos Santos, M., Lokman, P.M., 2018. Ovarian development of captive F1 wreckfish (hāpuku) *Polyprion oxygeneios* under constant and varying temperature regimes–implications for broodstock management. Gen. Comp. Endocrinol. 257, 86–96.
Yadetie, F., Arukwe, A., Goksoyr, A., Male, R., 1999. Induction of hepatic estrogen receptor in juvenile Atlantic salmon in vivo by the environmental estrogen, 4-nonylphenol. Sci. Total Environ. 233, 201–210.
Yamaguchi, T., Yamaguchi, S., Hirai, T., Kitano, T., 2007. Follicle-stimulating hormone signaling and Foxl2 are involved in transcriptional regulation of aromatase gene during gonadal sex differentiation in Japanese flounder, *Paralichthys olivaceus*. Biochem. Biophys. Res. Commun. 359, 935–940.
Young, G., Kusakabe, M., Nakamura, I., Lokman, P.M., Goetz, F.W., 2005. Gonadal steroidogenesis in teleost fish. In: Sherwood, N., Melamed, P. (Eds.), Hormones and their Receptors in Fish Reproduction. World Scientific Press, Singapore, pp. 155–223.
Zahangir, M.M., Rahman, M.L., Ando, H., 2022. Anomalous temperature interdicts the reproductive activity in fish: neuroendocrine mechanisms of reproductive function in response to water temperature. Front. Physiol. 13, 902257.
Zak, M.A., Manzon, R.G., 2019. Expression and activity of lipid and oxidative metabolism enzymes following elevated temperature exposure and thyroid hormone manipulation in juvenile lake whitefish (*Coregonus clupeaformis*). Gen. Comp. Endocrinol. 275, 51–64.