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Effects of temperature and sex steroids on sex ratio, growth, and growth-related gene expression in the Chinese giant salamander *Andrias davidianus*

Qiaomu Hu, Haifeng Tian, Hanbing Xiao*

Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Wuhan, Hubei 430223, PR China

ABSTRACT: *Andrias davidianus* is one of most farmed amphibians owing to its palatability and nutritional value. In this transitional group between aquatic and terrestrial animals the effects of temperature and sex hormones on sex ratio and growth are not fully understood. Here, we quantified the sex dimorphism of *A. davidianus* growth with adult males approximately 30% larger than females, and for the first time determined the time of initiation of sex differentiation to be ~98 days post-hatching (dph). Mortality increased significantly with increased temperature, from 9.4% at 20°C to 40.6% at 28°C. At temperatures ≥30°C we observed 100% mortality. The proportion of males was 66.1% at 28°C, significantly higher than in the control group ($p < 0.05$). 17α-estradiol induced larval feminization and produced female bias of 81–100% at concentrations from 25 µg l^{-1} to 1000 µg l^{-1}. However, sex bias was not observed in larvae exposed to 17α-methyltestosterone at concentrations of 50 µg l^{-1} and 100 µg l^{-1}. Additionally, growth characteristics at different temperatures showed that 28°C inhibited growth and 24°C promoted growth, reflecting the expression profile of growth-related genes (*GH*, *GHR*, and *IGF-1*). Sex steroids including 17β-estradiol and 17α-methyltestosterone significantly inhibited growth ($p < 0.05$). The results suggest that water temperature and sex steroids play a vital role in gonad differentiation and growth of *A. davidianus*.

KEY WORDS: Temperature · Sex steroids · Sex ratio · Growth · *Andrias davidianus*

1. INTRODUCTION

Sex is determined in vertebrates either genetically or environmentally, or via a combination of both factors (Nakamura 2009). In amphibians, when reared at natural temperatures, sex is generally genetically determined; however, environmental factors, including temperature, have been reported to affect sex (Wallace et al. 1999). Rearing at extremes of warm or cold temperatures may disturb sex differentiation and introduce a bias toward male or female. Among newts, *Triturus cristatus* larvae reared at temperatures of 14–26°C show a sex ratio of ~50:50. However, when temperature was increased to 28°C or decreased to 13°C, sex was significantly biased towards male and female, respectively (Wallace 1987). *Pleurodeles waltl* larvae reared at 32°C exhibited ZW female reversal to male (Dournon & Houillon 1984, 1985). Similar phenomena have been observed in *T. cristatus* (Wallace & Wallace, 2000) and the anurans *Bufo vulgaris* and *Rana temporaria* (Piquet 1930), *R. sylvatica* (Witschi 1929), and *R. catesbeiana* (Hsü et al. 1971). However, female reversal to male at high temperatures is not universal. Dournon & Houillon (1985) showed high temperatures to have the opposite effect, inducing ZZ male *P. poireti* reversal to female (Dournon & Houillon 1985).

Sex differentiation in amphibians is affected not only by temperature but also by sex steroids. In

*Corresponding author: xhb@yfi.ac.cn

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amphibians, sex steroid induction of the sex differentiation is complicated and shows species-dependent effects. Estrogen exposure is reported to produce all-female larvae of *P. waltl* (Gallien 1954), *Hynobius nebulosus* (Asayama & Miyamori 1957), *T. helviticus* (Gallien & Collenot 1960), and *Ambystoma punctatum* (Burns 1938, 1939). However, in *R. esculenta*, a high concentration of estrogen induced all-male offspring, while a low concentration produced all-female (Padoa 1936, 1943). Similar results have been observed in *R. pipiens* (Richards & Nace 1978) and *R. sylvatica* (Hayes 1998). Androgen has been reported to masculinize larvae in some anuran species (Ohta 1987), but showed no effect on others (Hayes 1998). In urodeles, androgen showed no effect on some tested species and resulted in paradoxical feminization in others (Hayes 1998, Wallace et al. 1999).

Temperature and sex steroids influence the sex differentiation and sex ratio in amphibians, but do they affect growth as well? In *R. chensinensis,* the optimum temperature is reported as 10–20°C with growth affected outside this range (Wang & Wang 2008). Growth rate of *R. chensinensis* increased with the temperature within a certain temperature range (Wang 2006), while extreme low temperature inhibited growth of *R. chensinensis* and *B. gargarizans* (Wang et al. 2005).

The Chinese giant salamander *Andrias davidianu*s, considered a living fossil that experienced little outward change during the past 350 million years (Gao & Shubin 2003), was historically widely distributed in many provinces of China (Yang et al. 2011). Due to environmental degradation and harvesting by humans the wild population has declined dramatically. Since the 1980s, the giant salamander has been classified as Endangered by the International Union for Conservation of Nature and Nature Resources. Due to its palatability and nutritional value the species is artificially bred and reared in China. To expand the market, the Chinese government has allowed trade of the giant salamander without a 'Manage and Exploit' or a 'Transport' license since 2015.

To determine whether temperature and sex steroids affect the sex ratio and growth, the present study (1) quantified sex growth dimorphism and (2) the developmental time of initiation of sex differentiation. The latter is important for inducing sex reversal to produce single-sex stock. Effects of temperature and sex hormones on *Andrias davidianus* larval sex ratio and growth were determined to aid in the captive rearing of these amphibians.

2. MATERIALS AND METHODS

2.1. Gonad development analysis

The experiments were carried out at the Zhejiang Yongqiang Chinese Giant Salamander Company (Jinhua, Zhejiang Province, PR China). To analyze the gonad development and identify the time at which sex differentiation began in *Andrias davidianus*, tissue containing the gonads was collected at 48, 62, 98 and 130 days post-hatching (dph), and gonads at 170, 210, 270, 360, and 545 dph and at 3 yr post-hatching. For each stage, 10–25 samples were fixed in 4% paraformaldehyde (pH 7.5) for 24 h and stored in 70% ethanol to identify the physiological sex by histology and to determine the time post-hatching of initiation of sex differentiation.

2.2. High temperature and sex hormone treatment

Based on the onset of sex differentiation for *A. davidianus*, a total of 800 larvae before sex differentiation (70 dph) were used to carry out the first-year experiment. Groups of 40 larvae in aquaria (1 × 1 × 0.3 m^3) were exposed to water at temperatures of 24, 28, and 32°C, increasing by 0.5°C h^{-1}, with a group held at 20°C as control. The water temperature was controlled by the temperature control system (SOBO). Total mortality was seen in the 32°C group within 2 d. A further 40 larvae exposed to water of 30°C exhibited total mortality within 5 d, while the other groups showed no or few deaths. Two series of experiments at temperatures of 20, 24, or 28°C were performed up to 8 mo post-hatching. In the second year, 1200 larvae at 55 dph, in groups of 200, were placed in aquaria containing water at 20, 24, and 28°C, with 2 aquaria per temperature level. Four series of experiments at each temperature were performed. Larvae were held until 8 mo post-hatching, after which salamanders were reared at ambient water temperature (20°C).

400 larvae, 40 per first-year group, were used to assess effects of sex steroid exposure. Dry powders of 17β-estradiol (E) and 17α-methyltestosterone (MT) were dissolved in 95% ethanol and then diluted in water. Larvae were immersed daily for 10–12 h in water containing E at 25 µg l^{-1} (E1), 100 µg l^{-1} (E2), 500 µg l^{-1} (E3), 1000 µg l^{-1} (E4) or MT at a concentration of 50 µg l^{-1} (MT1), 100 µg l^{-1} (MT2); the rest of the time the larvae were reared in water with no hormones. The trial was duplicated and continued to 8 mo post-hatching.

2.3. Mortality rate and sex ratio after high temperature and hormone treated

At the conclusion of the temperature trial, mortality was combined to analyze survival rate of each group. After high temperature treatment, when the exposed salamanders had been reared at ambient temperatures for 18 mo, the gonads were removed, fixed in 4% paraformaldehyde (pH 7.5) for 24 h, and stored in 70% ethanol to identify the phenotypic sex. Gonads and gonadal tissue were dehydrated through an ethanol gradient and cleared in xylene, embedded in paraffin, and 5–6 µm sections were cut and stained with hematoxylin-eosin (H&E), then examined under light microscopy (Olympus) to assess phenotypic sex.

Survival rate, mortality rate and sex ratio were calculated as follows:

$$\text{Mortality rate (MR) (\%)} = 100 \times n_m/n \quad (1)$$

$$\text{Average mortality rate (AMR) (\%)} = 100 \times n_t/n_y \quad (2)$$

$$\text{Survival rate (SR) (\%)} = 100 \times n_s/n \quad (3)$$

$$\text{Female proportion (FP) (\%)} = 100 \times N_f/N \quad (4)$$

$$\text{Average female proportion (AFP) (\%)} = 100 \times N_a/N_y \quad (5)$$

$$\text{Male proportion (MP) (\%)} = 100 \times N_m/N \quad (6)$$

$$\text{Average male proportion (AMP) (\%)} = 100 \times N_b/N_y \quad (7)$$

where n is the initial number of salamanders in each group; n_m is the number of dead salamanders in each group; n_t is the total number of dead salamanders at the same temperature from the 2 study years; n_y is the total number of salamanders at the same temperature in the 2 years. n_s is the number of surviving salamanders in each group. N_f is the number of females in each group; N_m is the number of males in each group; N is the total number of salamanders in each group; N_a is the total number of females in the 2 years; N_b is the total number of males in the 2 years; N_y is the total number of salamanders in the 2 years.

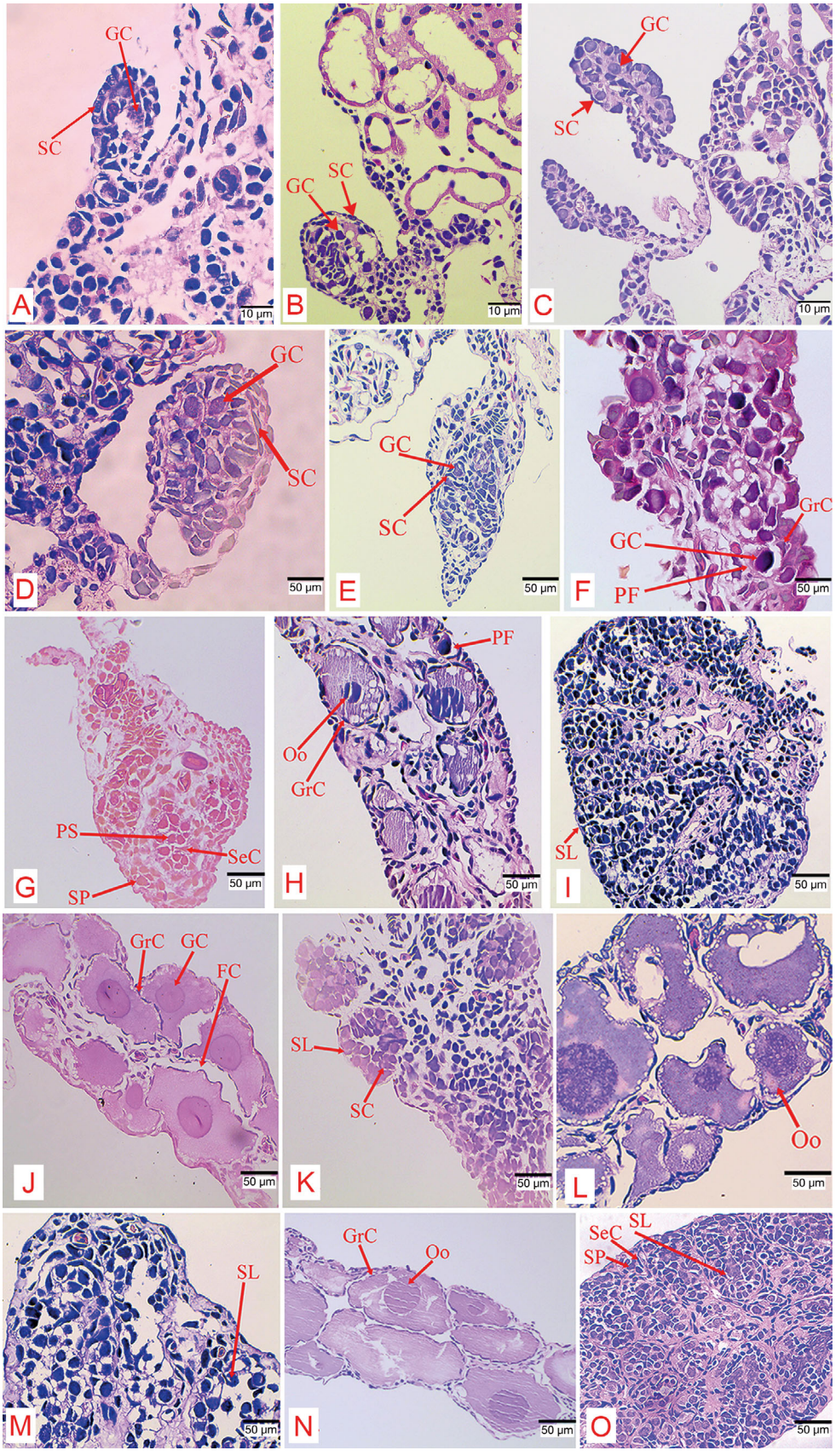
2.4. Growth characteristic analysis

To quantify sex dimorphism with respect to growth, 1173 broodstock salamanders with determined sex were weighed (age 9–12 yr), and the relative growth rate (RGR) was calculated according to the formula. Relative growth rate (RGR; %) = 100 × (W−w)/w (W: weight of males; w: weight of females). *Andrias davidianus* larvae were held at 18–20°C and fed on Chironomid larvae until 5 mo post-hatching, and then reared on chopped *Hypophthalmichthys molitrix* 1 feeding d^{-1}). Body weight and body length of at least 30 salamanders per stage were measured at 47, 98, 132, 164, 201, 230, 270, 285, 311, 345, and 353 dph, as well as 18 and 30 mo, and 3.5 yr post-hatching. At 98, 164, 201, 270, and 345 dph and 18 and 30 mo post-hatching, at least 3 individuals of each stage were killed after anaesthesia with MS222 according to Yangtze River Fisheries Research Institute Care Committee regulations (No. 2013001). The pituitary and brain were collected and stored at −80°C for extraction of RNA.

To detect the influence of high temperature and hormones on growth, the body weights and body lengths of at least 30 salamanders from the above study were measured at 98, 132, 164, 201, 230 dph during the temperature exposure and at 270, 285, 311, and 345 dph without the temperature treatment; body weight and body length of at least 30 randomly selected salamanders from each group was measured at 132, 164, 201, 230 dph during the hormone treatment and at 285, 311, and 345 dph without the hormone treated. To detect the expression profile, the pituitary and brain tissue were collected 201 dph (during exposure) and 270 dph (after exposure) in the temperature group and control group, respectively, and stored at −80°C for RNA extraction.

2.5. qRT-PCR analysis

The RNA was extracted using the TRIZOL method from brain and pituitary at 201 dph and 270 dph from the high temperature treated group and control, respectively, and then the total RNA was treated with RNase-free DNaseI (Tiangen) at 37°C for 1 h. Finally, cDNA synthesis was carried out as described in Hu et al. (2017). Quantitative real-time PCR (qRT-PCR) was conducted on a QuantStudio 5 real-time PCR system (Applied Biosystems) as described in Hu et al. (2015). mRNA expression for each gene was analysed at least in 3 samples and independently in triplicate. Based on previous reports (Yang et al. 2010, Ji et al. 2011), the growth hormone gene (*GH*) (pituitary), growth hormone receptor gene (*GHR*) (brain), and insulin-like growth factor-1 gene (*IGF-1*) (brain) were selected from the transcriptome database. Specific primers were designed according to the sequences (Table S1 in the Supplement at www.int-res.com/articles/suppl/b028p079_supp.pdf), and beta-actin was used as the internal reference to evaluate the relative expression level. The reaction was performed according to the QuantScript RT kit (Takara) manufacturer's instructions with 10 µl SYBR Premix


A
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SC
10 µm
B
GC
SC
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C
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D
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E
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F
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50 µm
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50 µm

Ex Tap, 0.4 µl ROX reference dye, 0.4 µl of each primer and 1 µl cDNA and made up to 20 µl using double-distilled water. The reaction was carried out as follows: 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C, with final construction of a dissociation curve. Pituitary and brain were obtained from 3 specimens in the temperature group at 201 and at 270 dph respectively, and relative gene expression was calculated.

2.6. Statistical analysis

SPSS 19.0 software (IBM) was used to analyse significant differences among the various groups. The weight differences between same age male and female adult *A. davidianus* was analyzed by independent samples *t*-test. When Levene test gave $p < 0.05$, signifcant differences were assumed. Conversely, equal variances were not assumed with $p < 0.05$. In 2-tailed tests, the 2 groups sampled were considered significantly different at $p < 0.05$. Differences of mortality rate and sex ratios at different temperatures and various hormone concentrations were analysed using chi-square test after data weight case followed by Fisher's exact test. Results were considered significant at $p < 0.05$. Difference in body lengths and body weights caused by temperature and hormone treatment and gene expression were analysed by one-way ANOVA, followed by Duncan's test. Results were considered significant at $p < 0.05$.

3. RESULTS

3.1. Gonad development

To observe gonad development and determine the time of sex differentiation, larvae of different ages were examined microscopically. At 48 dph, the germ cell was surrounded by somatic cells (Fig. 1A). At 62 dph, the size of the gonad had increased, with an increasing number of germ cells, but no structure changes (Fig. 1B). At 98 dph, the gonad was further increased in size, with the proliferation of germ cells and somatic cells, and the germ cells were dissociated from the cortex, suggesting beginning of sex differentiation (Fig. 1C). At 130 and 170 dph, (Fig. 1D,E) the quantity of somatic cell had increased. At 210 dph, the primordial follicle and spermatocyte were visible in ovary and testis, respectively. Germ cells were deeply stained and surrounded by granulosa cells (Fig. 1F). Testes at this stage contained spermatogonia and primary spermatocytes surrounded by Sertoli cells (Fig. 1G). At 270 dph, the oocyte was clearly visible in the ovary and a seminiferous lobule was formed in the testis (Fig. 1H,I). At 360 dph, the gonads were larger, and the shape altered from a thin thread to flat. Oocytes were larger, and the follicular cavity had formed (Fig. 1J). Oocytes and spermatogonia matured from 360 dph to 3 yr post-hatching (Fig. 1K–O).

Fig. 1. Histology sections of the developing gonads of *Andrias davidianus*. (A) 48 d post-hatching (dph); (B) 62 dph; (C) 98 dph; (D) 130 dph; (E) 170 dph; (F): ovary 210 dph; (G) testis 210 dph; (H): ovary 270 dph; (I) testis 270 dph; (J): ovary 360 dph; (K) testis 360 dph; (L) ovary 545 dph; (M) testis 545 dph; (N) ovary 3 yr old; (O) testis 3 yr old. GC: germ cell; SC: somatic cell; GrC: granulosa cells; PF: primordial follicle; SL: seminiferous lobule; SP: spermatogonia; FC: follicular cavity; SeC: Sertoli cell; PS: primary spermatocyte; Oo: oocyte. Scale bars are 10 µm in (A–C), 50 µm in (D–M), 100 µm in (N,O)

3.2. Mortality rate and sex ratio

Mortality rate was calculated relative to temperature (Table S2 in the Supplement). We found 100% mortality at 30°C (Fig. 2). The mortality rate was significantly higher at 24°C and 28°C (17.71% and

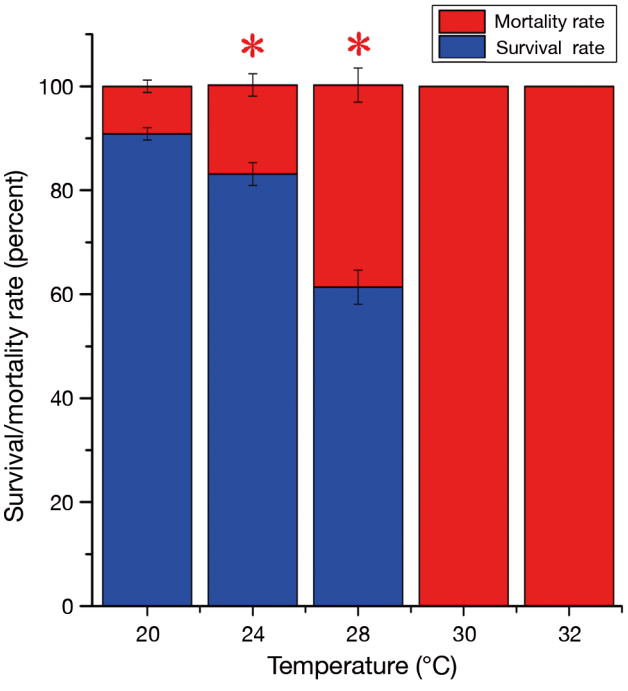

Fig. 2. Mortality rate of *Andrias davidianus* relative to water temperature. The red asterisk indicates a significant difference based on chi-square test between experimental and control groups ($p < 0.05$)

40.63%) than at 20°C (9.37%) (Table S2) ($p < 0.05$). At 28°C, the proportion of males was significantly higher (66.06%) (Table S3) than at 24°C (50%) and 20°C (50%) ($p < 0.05$) (Fig. 3A). The sex ratio with respect to hormone exposure showed that estrogen induced feminization, while methyltestosterone had no effect (Fig. 3B). Depending on concentration, the mean proportion of females in larvae exposed to estrogen ranged from 81.1–100% and was significantly higher than in the control group (50%) ($p < 0.05$) (Table S4). Additionally, in larvae treated with methyltestosterone sex ratios did not deviate from 1:1 (Table S4) ($p > 0.05$).

3.3. Growth analysis

Males grew more rapidly and to a larger size, increasing from (mean ± SE) 5.44 ± 0.73 to 9.13 ± 1.03 kg, compared to females that grew from 4.15 ± 0.42 to 7.01 ± 0.72 kg over the 4 yr study period (Table 1). Calculation of RGR showed males to significantly grow faster than females with ~30% at each period ($p < 0.05$).

Body weights and body lengths were monitored from 47 dph to 3.5 yr post-hatching. Mean body length increased from 5.12 ± 0.21 cm at 47 dph to 36.2 ± 2.86 cm at 3.5 yr post-hatching (Table S5) (Fig. 4A). From 47 to 345 dph, mean body weight gradually increased from 1.14 ± 0.15 to 33.75 ± 11.92 g, and subsequently sharply increased to 283.03 ± 61.20 g at 3.5 yr post-hatchingt (Table S5). Expression of *GH* in pituitary and *GHR* and *IGF-1* in brain exhibited a similar pattern, with a significant

Table 1. Body weight (± SE) of *Andrias davidianus* from ages 9 to 12 yr. N = number of specimens. *indicates significant difference ($p < 0.05$) between female and male weights by independent samples *t*-test. RGR: relative growth rate

Age (yr)	Female		Male		RGR
	N	Mean weight (kg)	N	Mean weight (kg)	
9	113	4.15 ± 0.42	84	5.44 ± 0.73	31.08 %*
10	185	5.28 ± 0.76	49	6.97 ± 0.86	32.01 %*
11	276	6.67 ± 0.88	68	8.81 ± 1.12	32.08 %*
12	263	7.01 ± 0.72	135	9.13 ± 1.03	30.24 %*

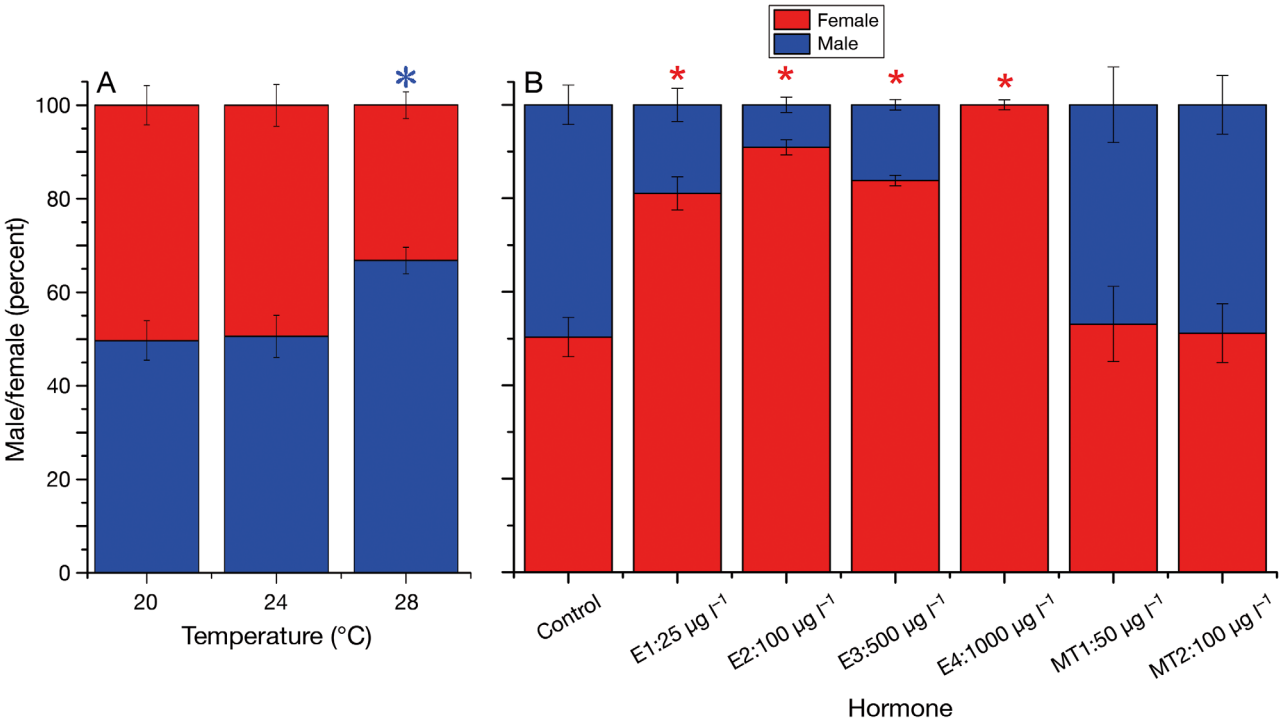

Fig. 3. (A) Effect of temperature on sex ratio of *Andrias davidianus*. (B) Sex ratio of *A. davidianus* after hormone treatment with estradiol or methyltestosterone. The blue asterisk indicates a significant difference in % males using chi-square test between experimental group and control group ($p < 0.05$). The red asterisk indicates a significant difference in % females using chi-square test between experimental group and control ($p < 0.05$). The *A. davidianus* larvae were treated by concentration of 17β-estradiol (E) and 17α-methyltestosterone (MT) as follows: E1: 25 μg l^{-1}, E2: 100 μg l^{-1}, E3: 500 μg l^{-1}, E4: 1000 μg l^{-1}, MT1: 50 μg l^{-1}, MT2: 100 μg l^{-1}

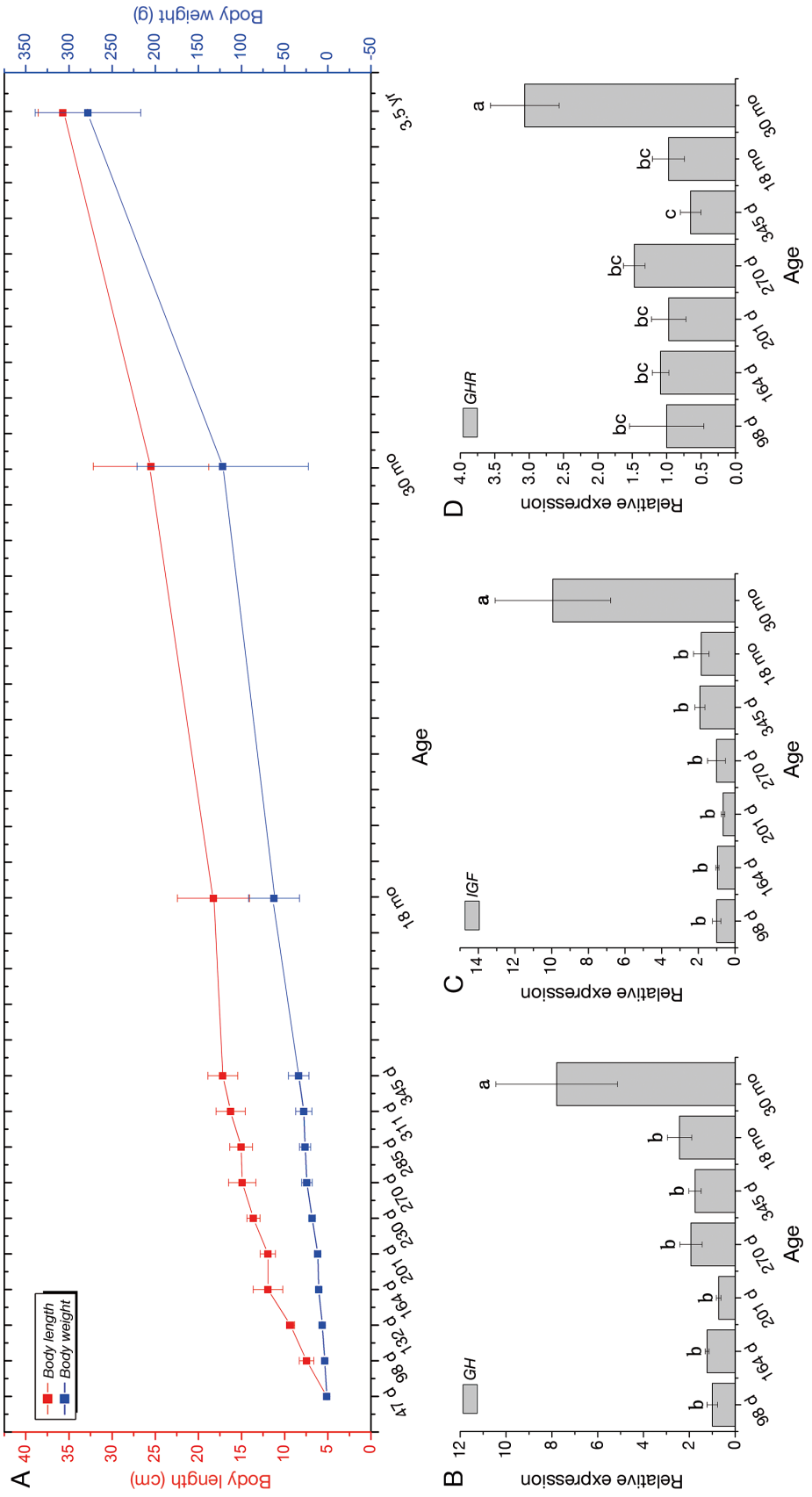

Fig. 4. Growth of *Andrias davidianus* relative to age. (A) Body length and body weight. (B) Expression of *GH* in the pituitary. (C) Expression of *IGF-1* in the brain. (D) Expression of *GHR* in the brain. Statistical analysis by 1-way ANOVA, followed by Duncan's test. Different letters indicate statistically significant differences ($p < 0.05$)

expression at 30 mo (Fig. 4) which was consistent with the weight increasing.

3.4. Effect of temperature and sex hormone exposure on growth

To assess the effect of temperature on growth performance, growth was measured during and following temperature exposures (Tables S6 & S7). Mean body length was significantly lower at 28°C than in the 20°C control group at the assessed ages ($p < 0.05$) (Fig. 5A). Mean body length at 24°C was greater than in the control group at 201 dph, but not significantly different ($p > 0.05$) (Fig. 5A). Body weight at 28°C was significantly lower than in the control group at all monitoring times ($p < 0.05$), except 132 and 345 dph. At 24°C, body weight was significantly greater than seen in the control group from 230 dph ($p < 0.05$). Body weight at 28°C increased slowly; when water was returned to ambient temperature at 240 dph, weight increased sharply, and no difference was observed between 240 and 345 dph ($p > 0.05$). Body weight at 24°C showed the opposite pattern from that at 28°C.

Mean body length of all hormone-exposed groups was significantly lower than in the control group from 164 dph ($p < 0.05$) (Fig. 5C). Body weight was also lower with sex hormone exposure, and a similar profile was exhibited (Fig. 5D). During hormone exposure, body length and especially body weight increased slowly. When hormone exposure ceased, body length and especially body weight sharply increased (Fig. 5D) (Tables S8 & S9).

3.5. Effect of temperature on growth-related gene expression

The expression level of growth-related genes was evaluated during and following temperature exposures. Expression of *GH* in the pituitary and *GHR* in the brain was significantly inhibited at temperatures of 24°C and 28°C ($p < 0.05$), while no difference was detected in *IGF-1* in the brain during the temperature trial ($p > 0.05$, Fig. 6). After cessation of the temperature exposure, the expression level of the 3 genes was significantly higher in the group formerly at 24°C ($p < 0.05$) than in the control group, while no significant difference from controls was observed in the former 28°C group ($p > 0.05$, Fig. 6).

4. DISCUSSION

The effects of temperature and sex steroid exposure on sex ratio and growth rate have been studied in many species (Chardard et al. 1995, Wallace et al. 1999, Nakamura 2009, Olmstead et al. 2010). How temperature and sex steroid exposure affect the sex ratio and growth rate of *Andrias davidianus* is unclear. In the present study, we monitored gonad development to determine the time of initiation of sex differentiation. The results showed proliferation of germ cells at approximately 98 dph suggesting the beginning of sex differentiation. In *Euphlyctis cyanophlyctis* gonad size was reported to have significantly increased at tadpole stage 27 due to the proliferation of germ cells and somatic cells, and the germ cell was pushed to the periphery. At this stage, gonad differentiation was evident by the initiation of meiosis in the oogonia (Phuge & Gramapurohit 2013). In *Xenopus laevis,* gonads were formed at stage 51 to 54 when the primordial germ cells (PGC) were migrating into the medullary region (Villalpando & Merchant-Larios 1990). 100% sex reversal was induced when estradiol benzoate was applied before translocation of PGC from gonadal epithelium into the medullary region (Villalpando & Merchant-Larios 1990).

In the present study, we found that high temperature induced individuals to become males. In the wild, the stage of sex differentiation generally occurs in winter, when the salamanders inhabit caves and subterranean rivers with an ambient temperature of 13–19°C. Studies of other species have shown that when larvae are reared outside their optimal temperature range the sex ratio is imbalanced. *Rana sylvatica* larvae reared at 32°C produced 50% males with the remainder exhibiting varying degrees of masculinized ovaries (Witschi 1929).

We observed sex ratio alterations in *A. davidianus* larvae exposed to sex hormones. In most anurans and all urodeles studied feminization is caused by estradiol but no masculinization by testosterone has been noticed (Wallace et al. 1999). *Pleurodeles waltl* larvae immersed in 50–100 µg l^{-1} estradiol became female or intersex, while low-dose testosterone had no sex reversal effect, but a dose exceeding 5 µg l^{-1} testosterone caused feminization or intersex (Gallien 1954). *Xenopus laevis* tadpoles exposed to 50 µg l^{-1} estradiol were all altered to female, although some lacked oviducts (Chang & Witschi 1955). In contrast, *Rana nigromaculata* and *R. japonica* can be masculinized by testosterone and are unaffected by estradiol (Wal-

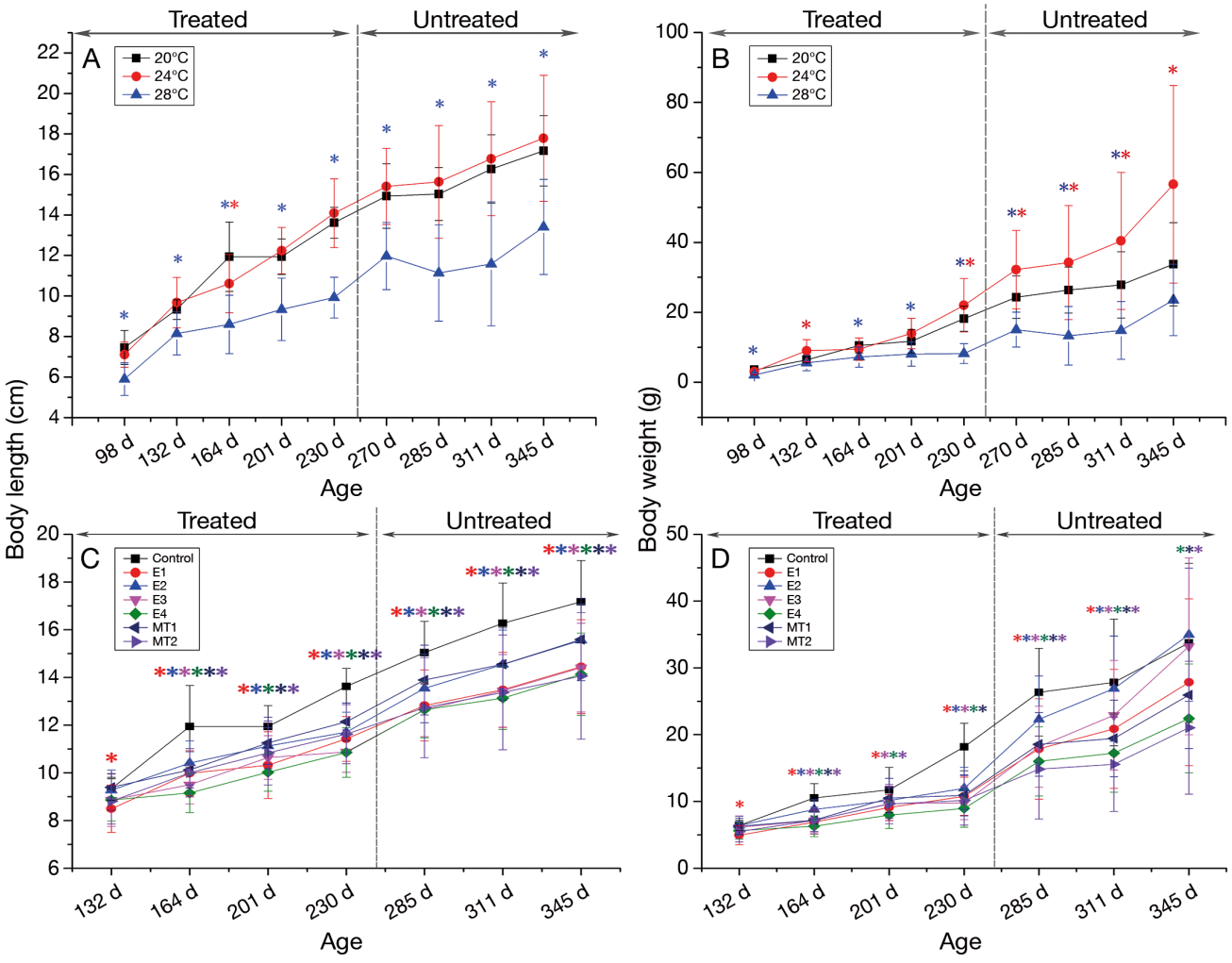

Fig. 5. Growth characteristics of *Andrias davidianus* at different temperatures and sex steroid exposure at different ages. (A) Body length of *A. davidianus* with temperature exposure. (B) Body weight of *A. davidianus* with temperature exposure. (C) Body length of *A. davidianus* with sex steroid exposure. (D) Body weight of *A. davidianus* with sex steroid exposure. Abbreviations as defined in Fig. 3. Different colored asterisks indicate statistically significant differences in growth performance using chi-square test between the corresponding experimental group and control group ($p < 0.05$)

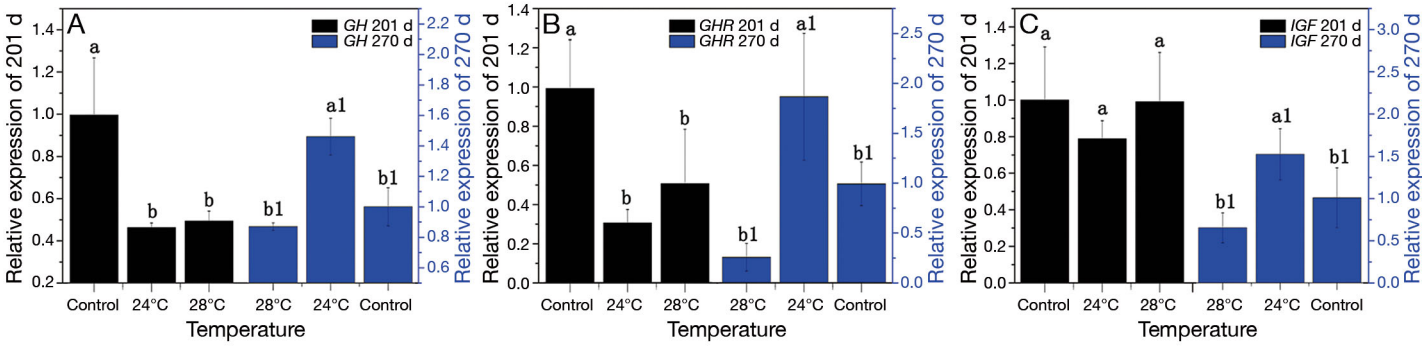

Fig. 6. Expression of growth-related gene during temperature exposure. (A) Expression of *GH* in the pituitary at 201 days post-hatching (dph, d) and untreated at 270 dph. (B) Expression of *GHR* in the brain at 201 dph and untreated at 270 dph. (C) Expression of *IGF-1* in the brain at 201 dph and untreated at 270 dph. Statistical analysis by 1-way ANOVA, followed by Duncan's test. Different letters indicate statistically significant differences at identical developmental stage but different temperature ($p < 0.05$)

lace et al. 1999). *R. pipiens* tadpoles can be masculinized by testosterone and feminized by estradiol (Richards & Nace 1978).

We observed growth dimorphism between male and female salamanders. In studies of Anura and Urodela, Shine (1979) estimated that in 530 of 589 frog species (90%) females are larger than males, males are larger than females only in 18 species (3%), and sexes are similar in size in 41 species (7%). In 79 salamander species females are larger than males in 48 (61%), males larger in 15 (19%), and no dimorphism is seen in 16 (20%) (Kupfer 2007). Many species exhibit a sex-dependent dimorphic growth pattern (Park et al. 2004, Chen et al. 2008). We found that growth-related gene (*GH*, *GHR*, and *IGF-1*) expression profiles were strongly consistent with growth characteristics, suggesting that the genes might be related to growth. The further growth of *A. davidianus* was assessed during and after the temperature trial. The results showed that growth was inhibited at a temperature of 28°C and promoted at a temperature of 24°C. In an earlier study (data not shown), we deduced the optimal temperature for salamander to growth to be 20°C according to the temperature of inhabited caves (~20°C), but there was no supporting experimental data. The present study provides the experimental data to estimate the optimal temperature for growth of *A. davidianus* at 24°C, which we expect to be the ideal temperature for farming of the giant salamander.

The expression of *GH* and *GHR* was significantly lower than in the controls at 24° and 28°C during the high exposure stage (at 201 dph). When the temperature was returned to ambient values (at 270 dph), a significant increase in *GH* and *GHR* expression was observed in the former 24°C group, but no difference was noted in the 28°C group. A temperature of 28°C was associated with lower growth and 24°C with higher growth. The expression pattern of *GH* and *GHR* was consistent with the growth profile. The expression of *IGF-1* was not significantly different from the control group during the high temperature exposure (at 201 dph), while the expression profile was similar to that of *GH* and *GHR* after the temperature was returned to ambient values (at 270 dph). The results suggested that *GH* and *GHR* were more strongly related to the growth of the salamander. These results have implications for the rearing of *A. davidianus*. At water temperatures outside the optimal range, feed consumption, enzyme activity, and feed conversion have been reported to be significantly affected (Wildhaber & Crowder 1990, Neverman & Wurtsbaugh 1994, Ji et al. 1996, Michalsen et al. 1998, Yee & Murray 2004). When energy intake exceeds energy expenditure, weight increases. Energy intake and expenditure are both affected by temperature (Diana 1995).

Sex hormones not only altered the sex ratio, but were also shown to inhibit growth. Growth was significantly inhibited during treatment and sharply increased when treatment ceased. A previous study reported that sex hormones affect body growth by modulating the *GH* signaling pathway (Fernández-Pérez et al. 2016). Estradiol and testosterone can modulate *GH* action by regulating pituitary *GH* secretion (Kerrigan & Rogol 1992, Mode & Gustafsson 2006). In mice, androgens may regulate the male skeleton through a stimulation of androgen receptors or estrogen receptors (Vidal et al. 2000).

In conclusion, the present study quantified *A. davidianus* growth sex dimorphism and determined the time of initiation of sex differentiation. Phenotypic sex and growth can be influenced by temperature and sex hormones during early development. Expression of growth-related genes agreed with the growth profile during and after treatment. Although the study did not elucidate the mechanisms involved in sex differentiation and growth in *A. davidianus*, the results reinforce the hypothesis that water temperature and some sex hormones (estradiol but not methyltestosterone) can modify both gonad differentiation and growth in Urodela. Further study to assess environmental factors and the underlying mechanisms is required to establish a rearing protocol for production.

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