Original Article

Modulation of phenotypic traits under different rearing temperatures: Experimental evidence in male guppy (*Poecilia reticulata*)

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Abstract: Climate change particularly global warming unceasingly imposes a selective pressure in many organisms that results in phenotypic plasticity particularly by expressing different adaptive phenotypes to shifting environmental conditions. The present study was, therefore, conducted to explore the phenotypic responses of male guppy (*Poecilia reticulata*), a popular model fish, to an ambient (28±0.91°C), high (32±0.12°C) and low (22±0.17°C) rearing temperature. Almost equal sized juvenile males were collected from the wild and reared up to 30 days maintaining necessary conditions similar among treatments except the water temperature. The findings revealed that high-temperature reared fish had significantly lower number of courtships, reduced survival, decreased body size, deformed body shape, limited colour patterns and reduced sperm bundle number than the ambient- and low-temperature groups. On the other hand, low-treatment males performed significantly higher number of courtships than ambient-temperature group, while ambient-temperature reared males possessed significantly higher body size and iridescent colour area than low- temperature treatment. Interestingly, the findings also revealed some trade-offs between traits under thermal-induced stressed conditions (i.e. both high and low temperatures). These findings elicit further information about the thermal condition dependent expression of phenotypic traits of fishes which infer about how fish species will adapt in the predicted changing aquatic environments because of unprecedented climate change.

Introduction

Global climate change is directly or indirectly altering the physical, chemical, and biological attributes of many aquatic environments which concomitantly influence the living animals particularly fishes (Whitehead et al., 2009; Whitney et al., 2016). In water bodies, the most implications of climate change mainly include rising temperature (Austin and Colman, 2007; Kaushal et al., 2010), increasing acidification (Zeebe et al., 2008; Koch et al., 2013), dwindling dissolved oxygen level (Ito and Momii, 2015; Bello et al., 2017) and changing water salinity (Ojaveer and Kalejs, 2005; Bonte and Zwolsman, 2010). Among these climate change variables, elevated water temperature is considered the most important one which can also trigger the fluctuation of other important abiotic factors such as dissolved oxygen, salinity, pH and some others (Roessig et al., 2004; Xiong et al., 2013; Bello et al., 2017). Thus, temperature has individual as well as synergistic effects on different traits of fishes (Pörtner, 2005; Wenger et al., 2016; Djurichkovic et al., 2019).

Fishes are ectothermic animals which solely depend on the external environment to regulate their body temperature. Any fluctuation in the surrounding's environmental temperature can create temperature-related stress which ultimately induces alternation in their physiological activities and thereby

affects directly or indirectly their behaviour, growth, reproduction, productivity and even survival. Examples include behavioral changes in brown trout, *Salmo trutta*, fry at both high and low temperatures (Colchen et al., 2016), growth reduction in Thai pangas, *Pangasianodon hypophthalmus*, reared beyond their optimal temperature range (Islam et al., 2019), decreased reproductive performance in damselfish, *Acanthochromis polyacanthus*, at a higher temperature (Donelson et al., 2014), and induced high mortality in sockeye salmon, *Oncorhynchus nerka*, at elevated temperature (Crossin et al., 2008).

When fishes encounter a fluctuating temperature, they try to acclimatize with this altered thermal condition through physiological mechanisms and behavioral modifications as a short-term response and genetic adaptation as a long-term response (West-Eberhard, 2003; Somero, 2010). Hence, these uncomfortable thermal tolerances cause the development of costly phenotypic plasticity (DeWitt et al., 1998) and/or local adaptation (Yampolsky et al., 2014). However, both phenotypic plasticity and local adaptation can be evolved together which are often very difficult to disentangle distinctly (Pulgar et al., 2005; Cottin et al., 2012). Studies have suggested that both adaptive mechanisms can be keys to determine the fate of a species or population exposed to a vulnerable elevated temperature (Somero, 2010; Wilson et al., 2019).

A number of studies have documented temperature-induced plasticity in behaviour (e.g. Oufiero and Whitlow, 2016), growth (e.g. Farrell and Franklin, 2016), survival (e.g. Crozier and Hutchings, 2014) and reproduction (e.g. Donelson et al., 2016) in different fish species. Evidence has also revealed these plasticity and local adaptations may also lead to trade-offs, i.e. specialist–generalist tradeoff (Angilletta et al., 2003) - the increment of performance of a trait at a certain range of temperature results in decrement of its performance in another range of temperature (e.g. Seebacher et al., 2015), or allocation tradeoff (Angilletta et al., 2003) - enhancing the performance of one or more traits at the expense of reducing the performance of another or more traits because of the

thermal variation (e.g. Hemmer-Brepson et al., 2014). Thus, fish can maintain the resource allocation strategy for the expression of target traits based on the available thermal condition. Most of these thermal-induced trade-off hypotheses have focused on other aquatic animals (Stillman, 2003; Pörtner et al., 2006; Armstrong et al., 2019) rather than specifically on fish.

It is unquestionable that the global temperature is rising which ultimately affects different fishes in various ways. Considering this issue seriously, a line of evidence has documented about the significant effects of elevated temperature on some selected traits in different fishes. However, given the potential negative impacts of global warming specially on fishes, more studies are needed addressing the short and long-term effects of temperature variation on various phenotypic traits. The present study, therefore, was carried out to investigate how different rearing temperatures could influence various phenotypic traits (e.g. courtship behaviour, survival, body size and shape, colour patterns and sperm bundle number) of a popular model species guppy (*Poecilia reticulate*).

Guppy is a small freshwater live-bearing and promiscuous fish (Houde, 1997). Males are typically smaller than females and exhibit highly complex colour patterns such as orange, iridescent and black spots. They also perform different kinds of courtship behaviour during matting to attract females (Houde, 1997). Their conspicuous colour patterns and exaggerated courtship behaviours are typically preferable to females during their mating (Kodric-Brown and Nicoletto, 2001). Studies have shown that males survival (Karayucel et al., 2006), body length (Breckels and Neff, 2013), body shape (Hendry et al., 2006), colour patterns (Grether et al., 2005), courtship behaviour (Endler, 1987), sperm size (Breckels and Neff, 2013) and some other traits (Karayucel et al., 2006; Burns et al., 2009) are strongly dependent on different ecological conditions. Therefore, the present study selected specifically the male of this popular model fish as the suitable experimental species to test whether different rearing temperatures could affect their various phenotypic traits.

Materials and Methods

Fish collection: Around 500 juvenile guppies were collected from different canals of Khulna city, Bangladesh. Then they were transported in oxygenated poly bag to the 'Wet Fish laboratory' of Fisheries and Marine Resource Technology Discipline, Khulna University, Bangladesh where the experiment was carried out. The collected juveniles were kept in three large glass aquariums (80×40×30 cm) and allowed to acclimatize with the existing laboratory water condition for one week.

Rearing conditions: From these collected stocks, all juvenile males were sorted out by observing their gonopodium. Finally, almost same sized juvenile males [total length (mm): 16.91±0.18 (mean±SE) and ANOVA: $F_{2,117} = 1.29$, $P = 0.28$] were selected to rear in aquariums (60×30×30 cm) under the three different experimental temperatures such as (1) ambient (28±0.91°C), (2) high (32±0.12°C) and (3) low (22±0.17°C) temperature. Each treatment consisted of two replications and each replication contained 20 randomly selected juvenile males from the stock. Thus, total 120 juveniles were reared in six replications of three treatments up to one month (30 days).

The high and low temperatures were chosen based on the annual mean daily maximum (31.3°C) and minimum temperature (21.9°C) in this region during the summer season (Yu et al., 2019). The water temperatures of all aquaria in high and low treatments were maintained by using individual thermostat (Shenzhen Yago Technology Co., Ltd., China), while the aquaria of ambient treatment were kept in natural condition without any temperature control. Every aquarium was covered with the styrofoam to minimize any temperature exchange.

Water quality management: Each aquarium was equipped with continuous aeration. About half of the water was exchanged once a week, and the faeces and uneaten food were removed by siphoning regularly. Adhered dirt inside the aquarium walls were also cleaned once a week. During the experimental period, dissolved oxygen (DO range: 4-7 mg/L and Kruskal-Wallis: $\chi^2 = 0.11$, $P = 0.95$) and pH (range: 7.3-8.9 and

Kruskal-Wallis: $\chi^2 = 0.15$, $P=0.93$) of water were monitored daily. To keep them in optimum range (Kithsiri et al., 2010; Shah et al., 2017), DO was maintained by providing sufficient aeration and if necessary, pH was maintained by adding diluted Sodium Hydroxide (NaOH) to increase and diluted Sulfuric Acid (H_2SO_4) to decrease the level (followed by Zahangir et al., 2015; Maoxiao et al., 2018).

Feeding: The experimental fishes were fed 6 days per week (once a day) with the commercial dry food (Wardley Total Tropical Gourmet Flake Blend[TM], the Hartz Mountain Corporation, Secaucus, NJ). Food was provided up to their satiation level throughout the experimental period.

Survival, body size and shape analysis: Fish was monitored every day and thereby, the number of survivals was recorded. At the end of rearing period, each male was narcotized for a while using the ice bath. The anesthetized fish was then placed on a laminated graph paper and captured a photograph maintaining a fixed distance (30 cm) using a digital camera (Canon DS126621). The narcotized fish recovered within a minute after transferring to its rearing water. Each image included a unique code so that the subsequent analyses of male traits were performed blind of treatment. The raw uncompressed images were imported into ImageJ software (v1.46) for the measurement of standard length (the distance in mm from the fish snout to the tip of its caudal peduncle).

The geometric morphometrics was used to estimate the variation in experimental male body size (centriod) and shape by following the methods described by Hendry et al. (2006). First, the tpsDig2 software was used to superimpose 16 landmark points on each image (see Fig. S1). Landmarks were subsequently designated as fixed (placed at homologous points on each image) or semi-sliders (placed on curved surfaces between fixed landmarks) using the tpsUtil software. Landmark data were subsequently analyzed using the tpsRelw v1.42 software to generate relative warp (RW) scores, which described shape variation as deviations from a consensus shape. The first three RWs were retained

finally as they collectively explained 74.35% of the overall variance in male body shape (hereafter referred to as RW_{1-3}). The remaining RWs were excluded from the final analysis because of their low explanatory potential (each explained <6%). The RWs are equivalent to the principal components in PCA (Adams et al., 2004). Finally, the shape variation captured by these RW scores was visually illustrated by using the MorphoJ software (Klingenberg, 2013).

Color patterns measurements: The ImageJ software was also used for the determination of different color patches of each experimental male. The total number of black, orange and iridescent spots was recorded (Nicoletto, 1993). The total area of each male's different colored spots on the left side of body was also measured (Brooks and Endler, 2001).

Sperm bundle number counting: In guppy, sperm are packaged in uncapsulated sperm bundles (also known as 'spermatozeugmata') containing almost equal number of sperm cells (~22,000) which do not differ significantly within individual ejaculates as well as across different males (Evans et al., 2003; Cattelan and Pilastro, 2018). The sperm bundles from each male were collected by following an established procedure (Evans et al., 2003). Briefly, sperm bundles were ejected from the individual anesthetized male of all treatments randomly by gently stripping on the abdomen. Then the number of sperm bundles was counted using an improved Neubauer haemocytometer under a microscope (LABOMED, Lx 400 microscope).

Courtship behavior observation: For behavioral tests, around 120 juvenile females (i.e. 40 individuals × 3 tanks) were reared separately in three large aquariums (80×40×30 cm) keeping them at the ambient temperature as 'stock females' which allowed to minimize the effects of temperature on females and their behavioral performances. After the above-mentioned sperm collection, they were kept for another week in their respective treatments so that sperm could be fully replenished for sexual interests (Pilastro and Bisazza, 1999; Gasparini et al., 2010). Then the temperature of high and low treatments was gradually adjusted with the ambient temperature (i.e.

increased or decreased at ±1°C per day). Once the temperature reached up to the ambient condition, a female from the stock was transferred in a pre-setup separate behavioral aquarium and allowed it to settle around 10 min. Then an experimental male was randomly chosen from a treatment and placed into the behavioral aquarium and allowed to settle for at least 5 min or until it showed sexual interest to the female (i.e. following the female or engaging in courtship). For each 10 min trial, the male mating behaviors were recorded as the number of sigmoid displays (males arch their body in a characteristic shaped posture and quiver), gonopodial thrusts (forced mating attempts in which males approach females from behind and attempt copulations without prior courtship or female solicitation), and the time (in seconds) spent by the male courting or chasing the female (a measure of the male's overall sexual interest in the female, hereafter "sexual interest") (Houde, 1997). After the trial, the tested male and female were kept separately in a labeled aquarium until the trials of all males were finished completely.

Statistical analyses: All analyses were performed using the 'R' software, version 3.6.1 (R Development Core Team, 2019). The descriptive statistics (means and SEs) were calculated using the 'pysch' package. The Shapiro-Wilk test of normality and the Levene's tests for homogeneity of variance were done with the 'onewaytests' package. Appropriate transformation methods were applied for non-normal data before their analyses. For examples, square roots were used for orange spot area and iridescent spot area, while natural logarithms were applied for black spot area and centroid size. The data of survival, courtship and color spot number were not used for normalization since they were considered as count data for other respective models.

The survival probability in each treatment (i.e. rearing temperature) was estimated with the Kaplan-Meier (K-M) model using the 'survival' package. In the model, the entire experimental period (30 days) was included as 'time' data, status of each fish (0 = alive and 1 = dead) as 'event' and treatment as 'predictor' variable. Then the Cox proportional

hazards (CPH) model was applied to find out the significance level of treatment effect on reared fish survival. The model also calculated hazard ratio (HR) of each treatment in which a HR above 1 indicates a covariate that is positively associated with the event probability (i.e. 1: no effect; < 1: reduced hazard and > 1: enhanced hazard).

The univariate analysis of variance (ANOVA) model was performed using the 'car' package to explore the effects of 'rearing temperature' (i.e. fixed effect) on fish standard length. Then, the univariate analysis of covariance (ANCOVA) models were applied for all traits (except for the counts and relative wraps data) where males' standard length was included as a covariate along with the fixed effect because some traits were strongly correlated with males' standard length. Then subsequent post-hoc (TukeyHSD) tests were performed using the 'multcomp' package to find out multi-comparison among different rearing temperatures.

During the geometric morphometrics data analysis, first a multivariate analysis of covariance (MANCOVA) model was run to test the overall effect of rearing temperature (i.e. fixed effect) on body shape (e.g. RW_{1-3}) and in the model, body size (log centroid size) was included as a covariate. Since the MANCOVA model revealed an overall significant effect of rearing temperature on RW_{1-3} (see below), subsequent ANOCOVA models were performed to identify where these effects lie.

For counts data analysis (e.g. courtship behavioral traits, color spot numbers and sperm bundle number), first the zero-inflation was checked by observing the distribution in histograms and then the 'glmmADMB' package was used to create the generalized linear mixed models (GLMM). In the models, the rearing temperature was entered as a fixed effect, males' standard length as a co-variate and individual male's ID as a random factor. The model's framework was formulated with the negative binomial distribution to approximate over dispersion (Lindén and Mäntyniemi, 2011). In the models, the zero-inflation option was adjusted based on having many zeros ('TRUE') or not ('FALSE') in the respective count variable.

To quantify the magnitude of effects of rearing temperature, the effect size was calculated based on the appropriate statistical significance tests. Finally, the Spearman's rho (ρ) was calculated and plotted using the "Performance Analytics" package to test for correlations among measured phenotypic traits to explore the associations and trade-off among them. All plots were made using the 'ggplot2' package to show the significant variations among treatments graphically.

Results

Survival performance: The survival analysis revealed that fish reared in high temperature had significantly reduced survival (mean±SE: 0.75±0.06 and HR = 5.7) than ambient (mean±SE: 0.95±0.03 and CPH: z = 2.24, $P<0.05$ and HR = 0.18) and low (mean±SE: 0.98 ±0.02 and CPH: z = 2.30, $P<0.05$ and HR = 0.09) treatment. The analysis also unveiled no significant variation in survival between ambient and low temperature rearing group (CPH: z = 0.56, P = 0.58; Fig. 1).

Body size and shape: After the experimental 30 days, it was found that the rearing temperature significantly influenced standard length (ANOVA: $F_{2,105}$ =15.128, $P<0.001$ and f = 0.53). The post-hoc (Tukey HSD) test showed that the males reared in ambient temperature (15.91±0.19 mm) had significantly larger standard length than the high (14.62±0.22 mm and t = 4.35, $P<0.001$) and low (14.51±0.20 mm and t = 5.04, $P<0.001$) treatments. However, there was no significant variation found between high and low treatment group (t = 0.37, P = 0.92; Fig. 2a).

The geometric morphometrics outcomes showed that rearing temperature had significant effect on males' body (centroid) size (ANCOVA: $F_{2,105}$ = 12.44, $P<0.001$ and f = 1.13). The post-hoc (TukeyHSD) test unveiled that males kept in ambient temperature had significantly larger centroid size than the low (t = 2.64, $P<0.05$) and high (t = 4.98, $P<0.001$) treatments, while males reared in low treatment showed larger centroid size than the high treatment (t = 2.67, $P<0.05$; Fig. 2b).

Table 1. The outcomes of MANCOVA and subsequent ANCOVA models to show the effects of rearing temperature and body (centroid) size on body shape (relative wraps, RW1-3). Significant values are shown in bold and italic.

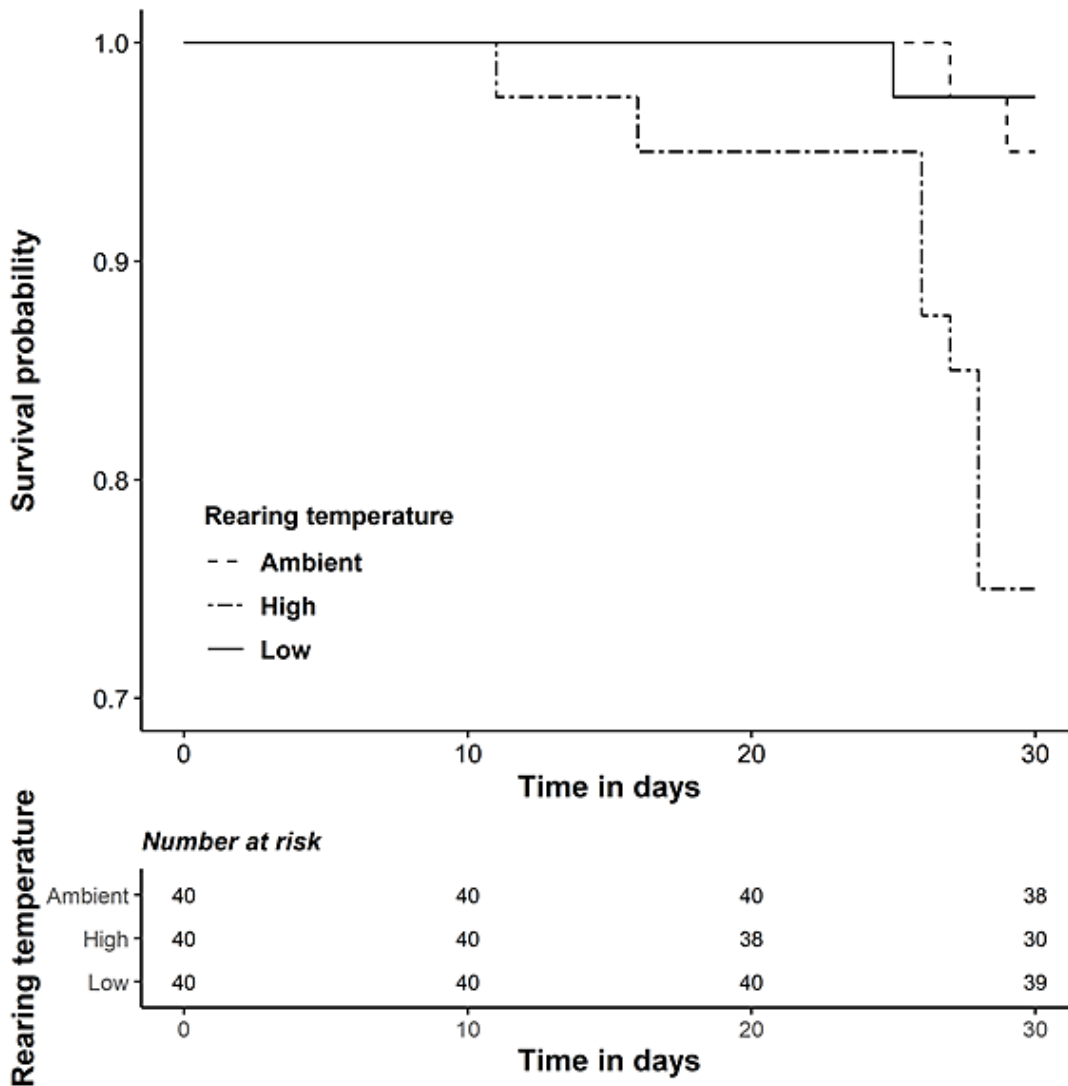| Model	Response	Predictor	test stat	F-ratio	df	*P-value*	R^2
MANCOVA	RW_{1-3}	Treatment	0.432	9.46	2,104	*<0.001*	0.18
		Centroid	0.163	6.63	1,104	*<0.001*	0.16
ANCOVA	RW_1	Treatment	0.04	7.67	2,104	*<0.001*	0.13
		Centroid	0.007	3.30	1,104	0.072	0.03
	RW_2	Treatment	0.008	7.07	2,104	*<0.01*	0.11
		Centroid	0.006	10.39	1,104	*<0.01*	0.08
	RW_3	Treatment	0.007	12.11	2,104	*<0.001*	0.18
		Centroid	0.001	4.55	1,104	*<0.05*	0.03

Figure 1. The Kaplan-Meier survival plot (top) and table (bottom). The plot shows the survival probability and table displays the survival record of experimental male guppy reared at different temperatures.

The MANCOVA and subsequent ANCOVA models revealed that treatment had significant effect on body shape (RW_{1-3}) (Table 1). The shape variation obtained by MorphoJ software in deformation grids further illustrated their differences because of treatment effect (Fig. S2). The plotted RW_1 revealed the variation in caudal region, i.e. elongation or shrinkage of caudal peduncle, RW_2 described variation in body depth and RW_3 showed the variation in abdomen (flank). Finally, the scatter plots of RW_1,
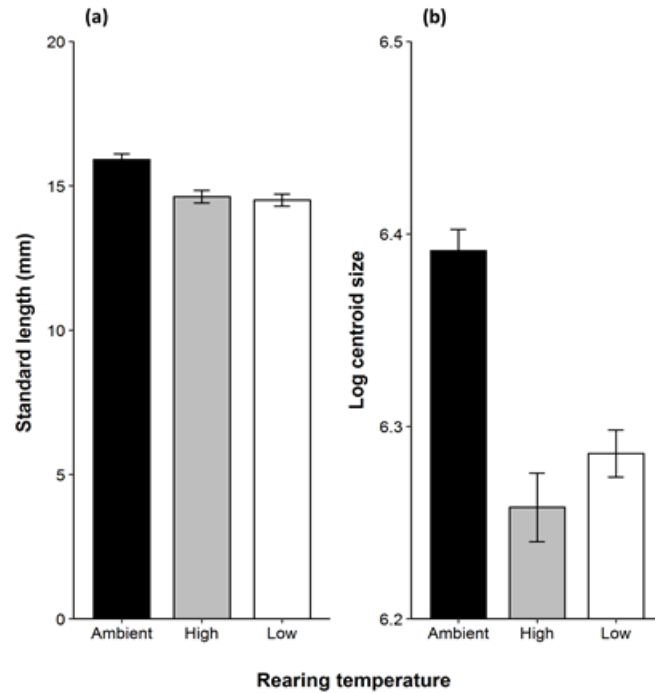
Figure 2. Effects of different rearing temperatures on (a) standard length and (b) body size (log centroid size) of experimental male guppy. Values are presented as mean ± standard error (SE). Differences in superscript letters indicate significantly variations ($P<0.05$).
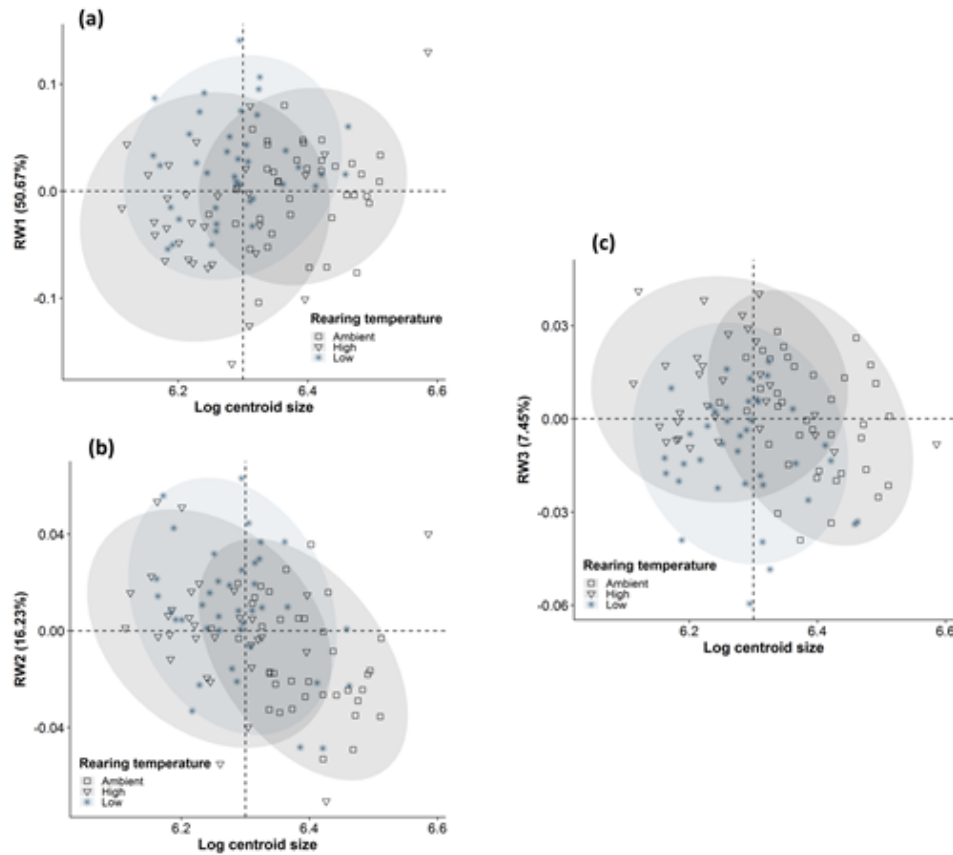

Figure 3. Relative wraps (RW1-3) as a function of body (log centroid) size of experimental male guppy reared at different temperatures. (a) RW1 scores represent the elongation or shrinkage of caudal peduncle, (b) RW2 scores describe the variation in body depth and (c) RW3 scores show the variation in abdomen (flank).
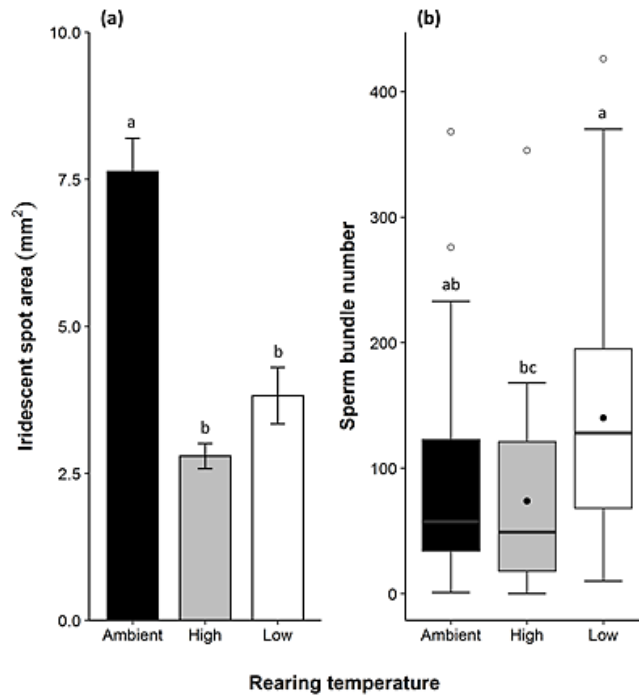
Figure 4. Effects of different rearing temperatures on (a) iridescent spot area and (b) sperm bundle number of experimental male guppy. Values are presented as mean ± standard error (SE). Differences in superscript letters indicate significantly variations (*P*<0.05).

RW_2 and RW_3 as a function of body (centroid) size clearly depicted how these shape scores varied because of temperature effect (Fig. 3a-c).

Color patterns: The analysis of color patterns revealed that the rearing temperature had significant impact only on the expression of iridescent spot area (ANCOVA: $F_{2,105} = 16.86$, *P*<0.001 and $f = 0.82$). The subsequent post-hoc (TukeyHSD) test showed that the ambient temperature group had significantly more iridescent area (7.63±0.56 mm^2) than the high (2.79±0.21 mm^2 and t = 5.62, *P*<0.001) and low (1.73±0.13 mm^2 and t = 4.37, *P*<0.001) treatments, while no significant variation was found between high and low (t = 1.56, *P* = 0.26; Fig. 4a) treatments. Except significant variation in iridescent spot area, the analysis did not find any significant effect on black spot number and area, orange spot number and area, and iridescent spot number.

Sperm bundles: The sperm bundle counting revealed that males kept in high temperature had significantly reduced number of bundles (73.71±13.64) than males reared in low temperature (138.10±15.724; GLMM: z = 2.08, *P*<0.05, R^2 = 0.09; Fig. 4b). However, no significant variation in sperm bundle number was

found between low and ambient (89.531±13.64 and GLMM: z = 1.60, *P* = 0.11) as well as high and ambient (GLMM: z = 0.35, *P* = 0.73) treatments.

Courtship behaviors: After the experimental period (30 days), the courtship trials showed that the high temperature reared males performed significantly lower number of sigmoid displays (0.45±0.08) than the ambient (1.84±0.63 and GLMM: z = 3.32, *P*<0.001) and low (6.08±0.82 and GLMM: z = 6.71, *P*<0.001) treatment males, while the ambient temperature group performed significantly lower number of sigmoidal displays than the low temperature treatment (GLMM: z=3.95, *P*<0.001, R^2 = 0.71; Fig. 5a).

Like sigmoid displays, the rearing temperature also significantly influenced the number of gonopodial thrusts where the high temperature group showed significantly reduced number of thrusts (0.19±0.07) than the ambient (1.0±0.29 and GLMM: z = 2.33, *P*<0.05) and low (6.08±0.82 and GLMM: z = 5.16, *P*<0.001) treatments. The courtship trails also unveiled that the low temperature group performed significantly higher number of thrusts than the ambient temperature group (GLMM: z = 2.47,
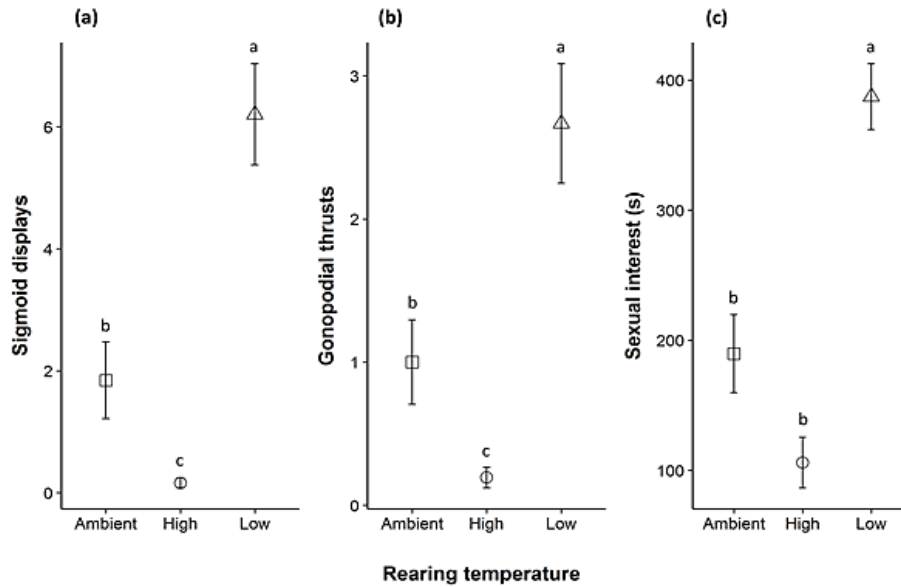
Figure 5. Effects of different rearing temperatures on (a) sigmoid displays, (b) gonopodial thrusts and (c) sexual interest of experimental male guppy. Values are presented as mean ± standard error (SE). Differences in superscript letters indicate significantly variations ($P<0.05$).
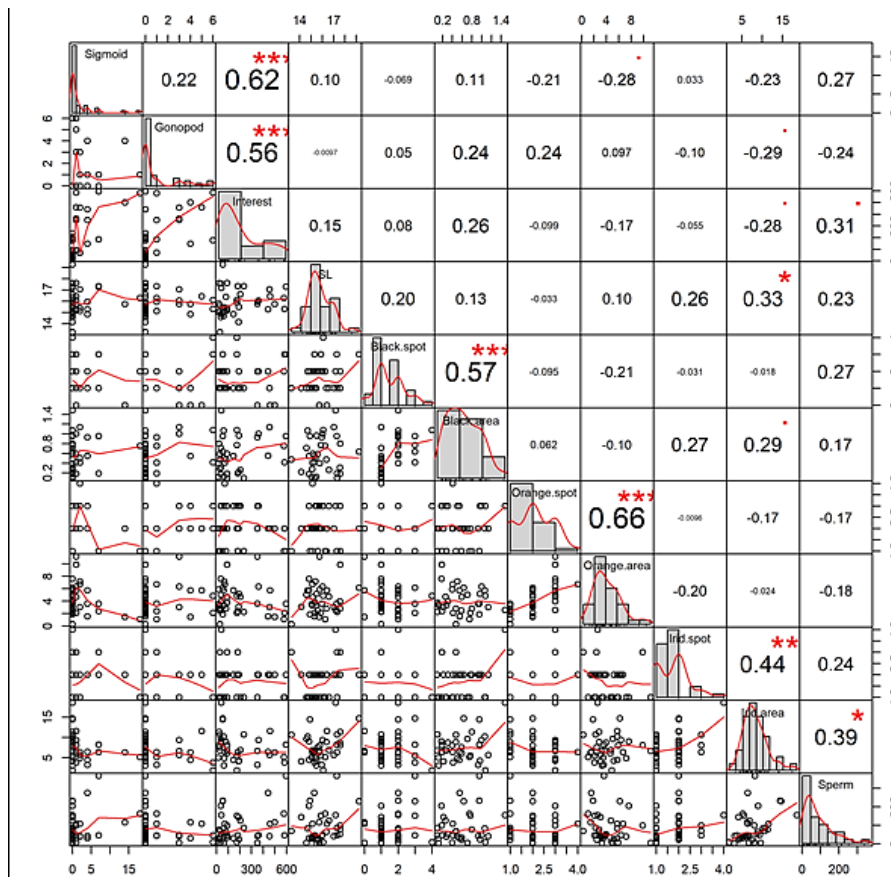

Figure 6. Correlation plot of multiple traits of experimental male guppy reared at ambient temperature (28±0.91°C). Here, shortened variables' full names are: Sigmoid- sigmoid displays number, Gonoopod- gonopodial thrusts number, Interest- sexual interest (sec), SL- standard length (mm), Black.spot- black spot number, Black.area- black spot area (mm2), Orange.spot- orange spot number, Orange.area- orange spot area (mm2), Irid.spot- iridescent spot number, Irid.area- iridescent spot area (mm2) and Sperm- sperm bundle number. Values around the axes levels are the range of unit of each variable. Correlation coefficients (ρ) are indicated with numeric values, while significance levels (P-value) are denoted by asterisks (*<0.05; **<0.01; ***<0.001).
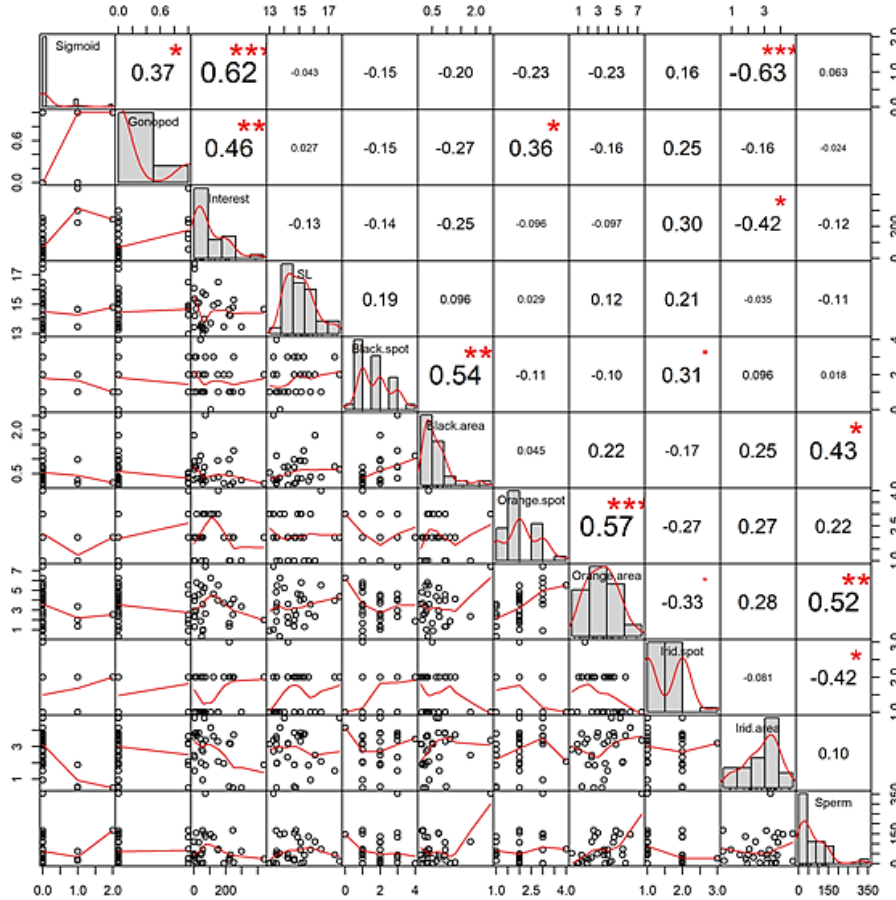
Figure 7. Correlation plot of multiple traits of experimental male guppy reared at high temperature (32±0.12°C). Here, shortened variables' full names are: Sigmoid- sigmoid displays number, Gonooopod- gonopodial thrusts number, Interest- sexual interest (sec), SL- standard length (mm), Black.spot- black spot number, Black.area- black spot area (mm2), Orange.spot- orange spot number, Orange.area- orange spot area (mm2), Irid.spot- iridescent spot number, Irid.area- iridescent spot area (mm2) and Sperm- sperm bundle number. Values around the axes levels are the range of unit of each variable. Correlation coefficients (ρ) are indicated with numeric values, while significance levels (P-value) are denoted by asterisks (*<0.05; **<0.01; ***<0.001).

$P<0.05$, $R^2 = 0.43$; Fig. 5b).

The analysis of time investment by males during the courtship trails disclosed that the high temperature reared males invested significantly shorter time (105.90±19.48 sec) than the low treatment (378.55±26.17 sec and GLMM: z = 5.51, $P<0.001$), while a marginally lower time was invested rather than the ambient temperature group (189.63±29.93 sec and GLMM: z = 1.76, $P = 0.07$). The experiment also revealed that the low temperature group showed significantly more sexual interest than the ambient temperature group (GLMM: z = 2.99, $P<0.01$, $R^2 = 0.38$; Fig. 5c).

Correlations among different traits: The correlation analysis revealed some significant positive and negative relationships among different traits in each

treatment. In ambient group, there were some significant positive associations found among different traits, while no negative association (e.g. indication of trade-offs) was emerged between traits. (Fig. 6). In high treatment group, significant positive associations were also found among various measured traits (Fig. 7). Interesting in high treatment, two negative associations were observed between sigmoid displays and iridescent spot area ($r = -0.63$, $P<0.001$), and sperm bundle number and iridescent spot number ($r = -0.42$, $P<0.05$) which are obvious evidence of trade-offs between these traits. On the other hand, the correlation tests for low treatment revealed significant positive associations among various traits, while only one trade-off was found between sperm bundle number and black spot number ($r = -0.41$, $P<0.01$;
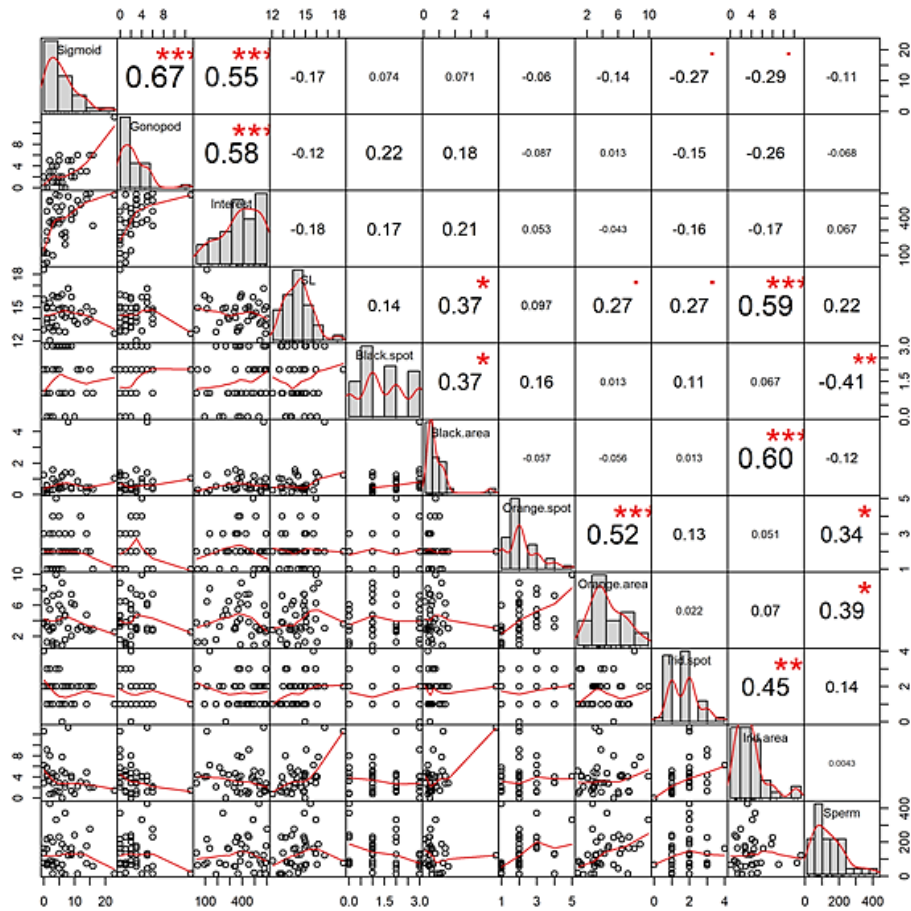
Figure 8. Correlation plot of multiple traits of experimental male guppy reared at low temperature (22±0.17°C). Here, shortened variables' full names are: Sigmoid- sigmoid displays number, Gonoopod- gonopodial thrusts number, Interest- sexual interest (sec), SL- standard length (mm), Black.spot- black spot number, Black.area- black spot area (mm2), Orange.spot- orange spot number, Orange.area- orange spot area (mm2), Irid.spot- iridescent spot number, Irid.area- iridescent spot area (mm2) and Sperm- sperm bundle number. Values around the axes levels are the range of unit of each variable. Correlation coefficients (ρ) are indicated with numeric values, while significance levels (P-value) are denoted by asterisks (*<0.05; **<0.01; ***<0.001).

Fig. 8).

Discussions

There is no doubt that global climate is changing and a large number of studies have prognosticated that global warming would be the main negative outcome of this unprecedented weather alteration (Flato and Boer, 2001; Knott et al., 2019). The rising of exiting water temperature is one of the major concerns because of this global warming (Morrill et al., 2005; Bello et al., 2017) which ultimately may affect the living aquatic organisms particularly many fish species directly or indirectly in different ways (e.g. Pörtner et al., 2001; Roessig et al., 2004; Crozier and Hutchings, 2014; Merilä and Hendry, 2014). A line of empirical evidence has also supported that elevated

water temperature can significantly affect fish behaviour (Colchen et al., 2016), growth (Islam et al., 2019), reproduction (Donelson et al., 2014), immunity (Kim et al., 2019) and their survival (Crossin et al., 2008).

During the present study, the survival rate in high treatment was significantly lower than the counter groups. Karayucel et al. (2006) have found that survival rates of gravid females and heat-treated fry of guppy signifiantly increased with increasing temperature (29°C and above), while Breckels and Neff (2013) have reported no significant mortality of guppy fry reared at different temepraures (23, 25, 28 or 30°C). The discrepancy between the findings of Breckels and Neff (2013) and other two studies (the present study and Karayucel et al., 2006) may be

because Breckels and Neff (2013) used 30°C as the highest temperature, while other two studies reared guppy above that temperature. Moreover, the variations in thermal rage of different locations and genetics might be other possible reasons for this different results. With different fishes, Rummer et al. (2014) have shown that six species of the equatorial reef fishes reduced significantly their aerobic scope and survivorship when they were reared for 14 days at 33 and 34°C which are above the existing thermal range of their habitats (29-31°C). In another study with the sockeye salmon (*O. nerka*), Crossin et al. (2008) have found that the mortality rate of high temperature (18°C) fish reared for 24 days was two times higher than the low temperature group (10°C). These studies confirmed that the potential physiological mechanisms were unable to produce the required amount of energy under very high thermal condition which might induce the significant mortality of fish exposed to higher temperature.

In the present study, males reared in ambient temperature obtained significantly higher standard length than their counter parts. This finding corroborates the outcomes of Breckels and Neff (2013) and Munoz et al. (2012). Breckels and Neff (2013) have found that the body length (standard length) of male guppy was significantly longer in 28°C than those of 25 and 30°C, whereas Munoz et al. (2012) reported that the body mass (body weight) of both male and female guppy was significantly lower at 30°C than those of other temperatures (23, 25 and 28°C). The possible reasons of significantly smaller body size (i.e. standard length and centroid size) of high temperature reared males might be because of extreme temperature impact on their physiological activities, restricted feed and feeding, and limited energy budget as discussed above. Under this adverse environmental condition, males perhaps utilized maximum energy to cope with the existing situation rather than the somatic growth. Indeed, this unfavourable thermal condition might increase energy demand for maintenance which resulted in decrease for the formation of new tissues for growth (Wood, 1932; Pepin, 1991). On the other hand, males reared

in low temperature had significantly lower standard length than the ambient group, while they had significantly higher body (centroid) size than the higher treatment fish. One plausible explanation could be that these lower-temperature exposed fish might invest available resources for body size development (i.e. body depth and bulkiness) rather than length increment (i.e. body elongation) which is very complex to interpret. Although the findings of geometric morphometrics also provided some evidence to support this outcome (see below), future studies are needed to expose the underlying physiological causes regarding this important issue.

Several studies have demonstrated that body size and shape of different fish species can be modified because of their adaptation mechanisms with the surrounding environments. For instances, changes in body size and shape have been revealed in *P. reticulata* because of habitats and predation (Hendry et al., 2006), *P. vivipara* due to salinity (Araújo et al., 2014), *Danio rerio* reared in different temperatures (Sfakianakis et al., 2011), *Perca fluviatilis* adapted to a warmer environment (Rowiński et al., 2015) and *Astyanax mexicanus* kept in different temperatures and turbidities (Corral and Aguirre, 2019). Moreover, Sheridan and Bickford (2011) have provided evidence of shrinking the body size of different animals, including fish in response to climate change. In the present study, high temperature reared fish possibly reduced their feed intake (Buentello et al., 2000), while the metabolic rate would be still rising (Jobling, 1997) which ultimately affected their overall growth and body size (Rahman et al., 2013), and the limited feeding and metabolism might also lead to have a variation in body shape plasticity (Wimberger, 1992). On the other hand, both high and low treatment males perhaps failed to utilize most of their consumed feeds for the development and energy yielding because of comparatively lower metabolic rate to cope with the extreme temperature (Johnston and Dunn, 1987; Sandersfeld et al., 2015). Their comparatively bulky body shape (RW_2) gives the notion of distended abdomen which might be because of their less physical movement or slower swimming

activities due to low temperature (Rome, 1995; Green and Fisher, 2004).

Male guppy having the most developed signals (e.g. colour patterns and courtship displays) usually achieve higher reproductive success (Kodric-Brown, 1985; Houde, 1997). Numerous lines of evidence support that the expression of sexual displays in male guppy exhibits condition dependence. For example, male courtship behaviour is highly sensitive to environmental conditions (Laudien and Schlieker, 1981; Rahman et al., 2013). There is also evidence that male colour patterns can vary depending on temperature (Breckels and Neff, 2013), light (Gamble et al., 2003), diet (Rahman et al., 2014), predation (Endler, 1991) and other ecological and environmental factors (Cole and Endler, 2015). Consistent with these findings, the present study also found that thermal stress (both high and low temperature) significantly reduced the total iridescent area indicating a thermal-induced oxidative stress in the experimental male guppy (please also see for other fishes: Lushchak and Bagnyukova, 2006; Jin et al., 2019). In accordance with the present finding, recent evidence has also confirmed that male guppy's iridescent colour area can be modulated under dietary stressed condition (Rahman et al., 2014; Evans et al., 2015). On the other hand, consistent with a previous study (Breckels and Neff, 2013), the findings of having no significant variation in orange colour (the indicator of 'good genes' of male guppy (Evans et al., 2004) could be because of its very high (Y-linked) heritability (Houde, 1992; Evans, 2010). Therefore, unlike iridescent colour, the reliability of this colour component is perhaps less compromised by thermal stress which has also been revealed under different environmental stressed conditions (Breckels and Neff, 2013; Evans et al., 2015). Similarly, the genetic linkage of black colour might be the strong reason of not having significant variation due to the thermal stress (Tripathi et al., 2009).

Extreme water temperature due to different reasons (e.g. global warming, manipulating rearing conditions, etc.) can interfere with the fish reproduction. For examples, elevated rearing temperatures (28 and 30°C) help to produce shorter and slower sperm than less warmed groups (23 and 25°C) in *P. reticulate* (Breckels and Neff, 2013), cold water (18°C) acclimated *Gambusia holbrooki* possess longer spermatozoa than the warm water group (30°C), while cold water males produce significantly fewer sperm cells than their counter group (Adriaenssens et al., 2012), and cold water (8°C) acclimated *Salmo trutta*'s spermatozoa produce faster but shorter flagella than their warm water (13°C) acclimated counterparts (Fenkes et al., 2019). Evidence with guppies have also shown that their sperm quality and quantity can be severely affected by dietary restriction (Rahman et al., 2013; Evans et al., 2015), predation (Devigili et al., 2019) and geographical variation (Evans and Magurran, 1999). It has been suggested that the thermal effects on sperm traits persist with local conditions which are species-specific (Breckels and Neff, 2013; Fenkes et al., 2019). Although the underlying mechanism of sperm depletion is still unknown, studies with different fishes have noticed that elevated temperatures can affect the reproductive hormones regulation, spermiation, blood concentration carrying steroid hormones and gonadal gene expression because of thermal-induced stress (Manning and Kime, 1985; Taranger et al., 2003; Pankhurst and King, 2010). Since the reproductive success of male guppy varies with sperm number (Boschetto et al., 2011), the findings of this present study ultimately indicate that the reproduction of this species could be negatively compromised with the warmer conditions.

In the present study, juvenile male guppies exposed to the high temperature (32±0.12°C) showed significantly lower number of courtships, higher mortality, reduced body size, deformed body shape, limited colour patterns and decreased sperm bundle number than the ambient (28±0.91°C) and low (22±0.17°C) treatments. Consistent with the present study, Laudien and Schlieker (1981) have demonstrated that immediate transfer to very low (20°C) and very high (30°C) temperature drastically reduced the sigmoid displays of male guppy. Observing the daily courtship behaviours of male

guppy, Reeve et al. (2014) have noticed that these activities were significantly dependent on temperature and light differently which were highest at dawn (mild temperature and light) and lowest during noon (very high temperature and light) and dusk (high temperature and very low light). Studies have suggested that elevated extreme water temperature can significantly affect fish neural system (Robertson and Money, 2012), cognitive ability (Toni et al., 2019) and olfactory sensitivity (Bhatt et al., 2002). It has been recently identified in the brain of male zebrafish where the olfactory receptors, 'PGF2α' (also known as pheromone complex), are responsible for eliciting courtship behaviour (Yabuki et al., 2016). The thermal-induced stress can disrupt the chemical communications between potential mating partners of fish using pheromones (Groot and Zizzari, 2019) which are the essential key components to trigger the courtship displays (Keller-Costa et al., 2014). Thus, the pheromone signals in the high treatment of present study could be affected which might reduce their courtship performances drastically.

During the courtship displays, fish need to swim exaggeratedly to exhibit the quality of performance to attract their mating partners. However, extreme thermal stress can reduce fish swimming and locomotion (Yuan et al., 2017) because induced thermal stress can reduce feed consumption and utilization (Buentello et al., 2000), hamper metabolic activities (Sandersfeld et al., 2015), provide less energy (Anacleto et al., 2018) and enhance oxidative stress (Nakano et al., 2014). Thus, the males reared in high temperature might suffer these problems which reduced their courtship performances during this study. On the other hand, the temperature of ambient treatment (28±0.91°C) in this study was still very warmer than the low treatment (22±0.17°C), and lower than the extremely high temperature (32±0.12°C). The findings of courtship trials also confirmed that this ambient temperature might still create some thermal stress which significantly reduced their courtships than the low treatment, but comparatively higher than the high treatment.

The overall findings of this present study showed significant effects of rearing temperatures on behavioural, physiological and reproductive traits. However, the behavioral performances, ornament expressions and reproductive outcomes responded differently to thermal variability in which males showed the allocation trade-offs (Angilletta et al., 2003) between few traits. Previous studies have revealed the evidence of trade-offs because of thermal stress between growth and reproduction in *Oryzias latipes* (Hemmer-Brepson et al., 2014), growth and survival in *O. myskiss* (Mogensen and Post, 2012), somatic growth and lipid storage in *Anoplopoma fimbria* (Sogard and Spencer, 2004), and sustained and burst swimming in *Menidia menidia* (Billerbeck et al., 2000). The possible reason of these trade-offs could be the resources allocation strategy of adapted fish among various traits under thermal-induced stress conditions. However, the underlying mechanism of these trade-offs are still unresolved and just only speculative and therefore, further research is needed to explore the physiological mechanisms which cause these trade-offs in fishes under stressed conditions.

Conclusion

There are many reports, discussions, documents, etc. to warn us confirming that global temperature is rising and if it continues in the present trend, it will create a severe stress to the living animals. Fishes are the most vulnerable among the affected animals as their physiology and its related activities entirely depend on surrounding environmental temperature. Therefore, this study was conducted to investigate how behaviour, growth and reproduction can be affected by different rearing temperatures in a model freshwater small fish, guppy (*P. reticulata*). The overall results showed alarmingly that the elevated temperature significantly reduced behavioral performances, retarded body size, deformed body shape, limited colour expression and reduced sperm production. The study also revealed few allocation trade-offs under thermal-induced stress conditions (both high and low temperature). Taken together, the findings suggest that global warming can severely affect our existing fisheries resources. Therefore, necessary strategies

must be taken urgently to save our valuable fisheries resources and further research should be carried out to explore how fish can adapt with the predicted elevated temperature and what are their adaptive physiological mechanisms that fish can maintain to cope with the unprecedented extreme thermal conditions.

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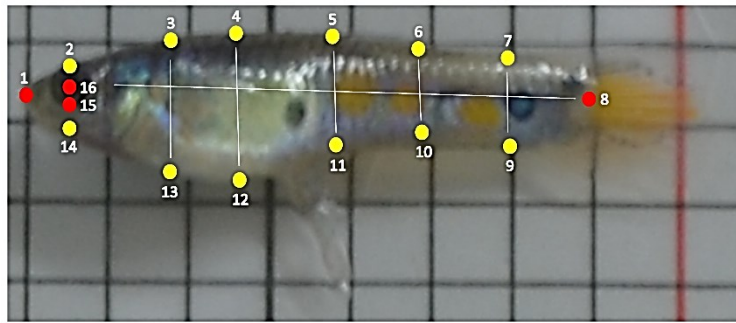
Figure S1. Identification of landmarks used in the geometric morphometric analysis. In the study, four fixed (red dots) and 12 sliding (yellow dots) semi-landmarks were positioned on each image using the tpsDig2 software. The four fixed landmarks were positioned on tip of snout (landmark 1), end of caudal peduncle (8), bottom of eye orbit (15) and middle of eye orbit (16). The remaining landmarks (2-7 and 9-14) were placed where the vertical lines of the standardized grid intersected the outline of the fish.
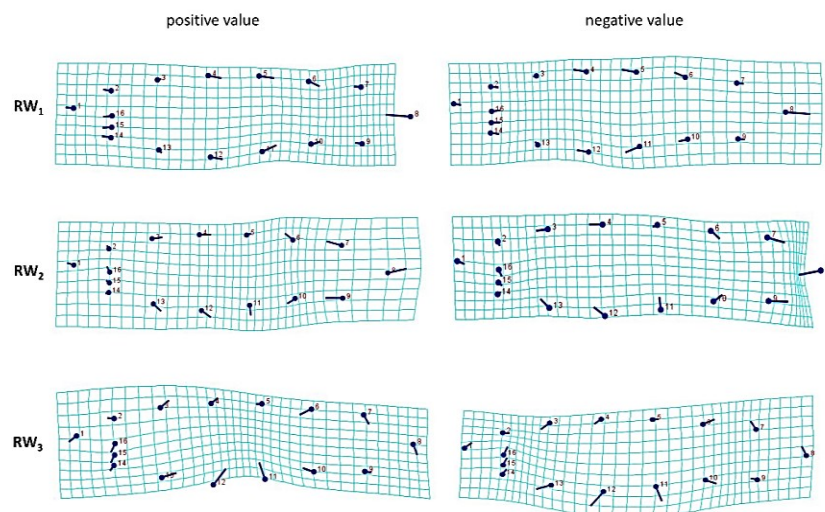

Figure S2. Effects of rearing temperature on the body shape variation of the whole sample (N=107). Deformation grids are associated to the most positive (left) and negative (right) values of the first three relative wraps (RW1-3) obtained by the thin-plate spline transformations from the TPSREGR software together with the MorphoJ software. Each lollipop denotes the position of each landmark and the shapes are shown in the grids with dots, and the shifts of landmarks to the target shape are indicated by lines.