Journal of Thermal Biology 81 (2019) 154–161

Contents lists available at ScienceDirect

Journal of Thermal Biology

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

Native amphibian larvae exhibit higher upper thermal limits but lower performance than their introduced predator *Gambusia affinis*

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ARTICLE INFO

Keywords:
Thermal tolerance
Thermal performance
Amphibian
Tadpole
Invasive species
Optimal temperature

ABSTRACT

Information on the thermal limits and physiology of ectothermic amphibians is crucial to our understanding of their ecology in the natural environment, particularly with predicted global changes in climate. We documented the thermal limits of larvae of three amphibian species native to Hong Kong, and their introduced, invasive predator, the mosquitofish (*Gambusia affinis*). We then used larvae of the brown tree frog *Polypedates megacephalus* as a model amphibian to further investigate growth, oxygen consumption rate and heat shock protein expression with changes in thermal regime. We found that *G. affinis* was the most tolerant of low temperatures but also the least tolerant of high temperatures. Despite the higher thermal tolerance of the amphibian larvae, further investigation on *P. megacephalus* demonstrated that optimal temperatures for physiological performance fall within a range of 18.0–21.6 °C, which is far lower than its upper thermal limit, implying that thermal stress occurs during part of the larval stage under natural environmental conditions. This could mean a reduction in their capacity to deal with other stressors such as pollution and predators, and that *G. affinis* may have an advantage over native amphibians.

1. Introduction

As with other ectotherms, amphibian physiology, behaviour and ecology are strongly associated with the surrounding thermal environment (Wells, 2007). Aquatic amphibian larvae are confined to the body of water they inhabit. As such, their body temperature is tightly linked to water temperature, and thus, temperature is believed to exert the greatest influence of all environmental factors on their biology and ecology (Ultsch et al., 1999). Many studies on ectotherms have identified temperature-dependent effects on a variety of physiological processes, such as growth and development (Lillywhite et al., 1973; Smith-Gill and Berven, 1979; Vondracek et al., 1988), and oxygen consumption and metabolic rates (Clarke and Johnston, 1999). In addition, behaviours, such as feeding rates (Lillywhite et al., 1973) and locomotory capabilities (Hirano and Rome, 1984; Huey and Stevenson, 1979), are also affected by temperature.

A simple linear relationship sometimes exists between physiological responses and temperature, as in studies on the effect of temperature on oxygen uptake (Clarke and Johnston, 1999; Feder, 1982) and heart rate (Harri and Talo, 1975) in some amphibian and teleost fish species, and specific growth rate, brood size, and larval mass of the mosquitofish *Gambusia affinis* (Vondracek et al., 1988). A strong relationship between body condition and mean water temperature has also been reported for the pond-breeding toad *Bufo bufo* (Reading, 2010). However, a non-linear response is more commonly found and a thermal performance curve has been used to describe general effects of temperature on thermal performance of ectotherms (Schulte et al., 2011). In many cases, ectotherms exhibit increased performance at an optimal temperature (T_{opt}) somewhere between their lower and upper thermal limits, from which performance starts to decline on either side of the temperature scale (Lillywhite et al., 1973; Stevens, 1988; Whitehead et al., 1989). An oxygen- and capacity-limited model was developed by Pörtner (2010) to explain the underlying processes that lead to temperature-dependent performance patterns for aquatic ectotherms. The model suggests that the aerobic scope of an aquatic ectotherm is limited by the availability of dissolved oxygen in the environment and the capability of the organism's circulatory and ventilation systems to balance supply of and demand for oxygen. Aerobic scope is maximized at T_{opt} when oxygen supply is more than sufficient to meet the basal demand and allows various activities such as foraging and courtship to be performed. However, with offsets between supply and demand as temperature decreases or increases from T_{opt}, the organism will

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https://doi.org/10.1016/j.jtherbio.2019.02.014
Received 8 February 2018; Received in revised form 31 January 2019; Accepted 23 February 2019
Available online 25 February 2019
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increase its anaerobic capacity and rely on molecular protection by heat shock proteins (Pörtner, 2010).

Temperature can alter physiological responses and induce behavioural changes in amphibians, which in turn may induce ecological changes at the population or community level, and these effects may be offset under predicted changes in climate. Evidence suggests that persistence of extant amphibian populations will be threatened under predicted global climate change scenarios (Blaustein et al., 2010; Carey and Alexander, 2003). For instance, amphibian immunity is negatively affected by changing temperature, and thus, climate change could potentially exacerbate amphibian population declines associated with emerging infectious diseases (Raffel et al., 2006). The occurrence of the chytridiomycete fungus, *Batrachochytrium dendrobatidis*, the pathogen responsible for an emerging disease that had caused massive die-offs in some amphibian populations, was significantly associated with climate change, and rising temperature was specifically linked to its occurrence (Bosch et al., 2007). Under current climate projections, it is predicted that European amphibian species will not be able to cope with the warming climate (Araújo et al., 2006). This effect will be amplified for highly fragmented and degraded habitats because of amphibians' low dispersal capacity (Araújo et al., 2006). Shifts in breeding phenology with warmer spring temperatures were documented for four of six frog species in the US (Gibbs and Breisch, 2001) and for common toads (*Bufo bufo*) in the UK (Reading, 1998), and reduced fecundity in female wood frogs (*Lithobates sylvaticus*) was observed in warmer winters (Benard, 2015). Furthermore, the negative effects of invasive species could be exacerbated by the changing climate (Hellmann et al., 2008). Aquatic invasive predators are often ectothermic, which means that they may increase their food consumption rate to cope with an increase in metabolic rate as temperature rises (Rahel and Olden, 2008). This may alter predator-prey dynamics in aquatic ecosystems.

Despite increasing research on the effects of climate change on amphibians, particularly in North and South America and Europe, studies on Asian species remain scarce. Given variation in latitudinal extent of species' distributions and habitat use, there is high heterogeneity in the physiology and ability of different amphibian species to cope with thermal stress (Brattstrom, 1968; Cupp Jr, 1980; Dunlap, 1968), and such variation may result in different ecological responses. In particular, amphibians in tropical areas are more likely to be affected by climate change given their relatively higher sensitivity to temperature change and that they are already living in an environment close to their optimal temperatures (Deutsch et al., 2008).

Understanding how amphibian physiology responds to different temperatures will provide baseline information on their thermal ecology and allow for assessment of their vulnerability to global climate change (Huey et al., 2012), as well as how temperature may interact with other stressors in their environments, such as invasive species. In this study, we examined a number of physiological endpoints, including thermal tolerance, growth performance, oxygen consumption rate and expression of stress proteins.

The objectives of this study were to: 1) establish baseline levels of thermal tolerance for the larvae of three tropical Asian amphibian species and their invasive predator *Gambusia affinis*; 2) use one amphibian model species to investigate how physiology responds to temperature; and 3) compare the thermal physiology of amphibians and *G. affinis* to better understand how changing climate will affect their interactions.

2. Methods

2.1. Target species

This study focused on lowland, wetland-breeding amphibians that are likely to experience temperature extremes with climate change predictions and that also co-occur with *G. affinis*: Asian common toad (Bufonidae: *Duttaphrynus melanostictus*), marbled pigmy frog (Microhylidae: *Microhyla pulchra*) and brown tree frog (Rhacophoridae: *Polypedates megacephalus*). All three amphibians are listed as 'Least Concern' by the IUCN (Lau et al., 2009; van Dijk et al., 2004a, 2004b).

Duttaphrynus melanostictus is broadly distributed in South, East, and Southeast Asia, spanning from northern Pakistan west to Taiwan, with its southern limit reaching parts of Indonesia (van Dijk et al., 2004a). It is the only explosive breeder found in Hong Kong with the first breeding event peaking in the beginning of the rainy season in February and a second peak two to three months afterward. It lays strings of eggs primarily in lentic waters, although eggs are occasionally observed in lotic systems. Their tadpoles are benthic grazers (Lau, 1998).

Polypedates megacephalus is distributed throughout South and East Asia from India to China, including Hong Kong, Macau and Taiwan (Lau et al., 2009). This species breeds throughout the rainy season and constructs a foam nest to contain its eggs. These nests are usually attached to vegetation from a few centimetres to several meters above the water surface. *Polypedates megacephalus* breeds primarily in lentic habitats, but also occasionally in lotic waters (Lau, 1998). Larvae of this species are benthic grazers but also spend a lot of time in the water column, potentially filter feeding on suspended algae.

Microhyla pulchra is distributed in South China, Cambodia, Laos, Thailand and Vietnam (van Dijk et al., 2004b). The species' breeding season spans the entire rainy season during which they lay eggs in films of a single egg thick on the surface of standing water bodies. This species breeds exclusively in lentic waters (Lau, 1998) and its tadpoles are suspension feeders which spend most of their time at or near the water surface.

Globally, *G. affinis* is considered one of the top 100 most detrimental invasive species ((GISD) Global Invasive Species Database, 2010). It is native to North America but was widely introduced for control of mosquitoes around the globe. Once established, it competes with or preys on native species and thereby offsets local ecological balance (Blaustein and Karban, 1990; Keskin, 2014; Komak and Crossland, 2000). This species possesses traits shared with other invasive species that aid in their success as invaders, such as high fecundity, high tolerance to environmental changes, and aggressiveness (Pyke, 2005, 2008). Diamond (1996) described this species as an "A-bomb" (referring to an atomic bomb) for its impacts on California newt (*Taricha torosa*) populations. *Gambusia affinis* was first introduced to Hong Kong as a biological control for mosquitoes in the 1940s and has since established local populations across Hong Kong (Dudgeon and Corlett, 2004). In Hong Kong, *G. affinis* has been documented to prey on five species of native amphibians (Karraker et al., 2010; Lau, 1998) and may, therefore, threaten their populations.

Permissions of the Department of Health, the Government of the Hong Kong Special Administration Region (Ref Nos. (11–189) in DH/HA&P/8/2/3 Pt.27 and (10–147) in DH/HA&P/8/2/3 Pt.19) had been obtained for the use of animals in the experiment.

2.2. Sampling site

Amphibian egg masses and adult mosquitofish were collected from Long Valley, Hong Kong Special Administrative Region, China (22°507′N, 114°113′E) between February and September in 2011 and 2012 (from the first rain prior to the wet season through the end of the wet season). Long Valley is one of the largest remaining wet agricultural areas in Hong Kong and like many agricultural areas in South China, Long Valley is heavily infested with exotic, predatory species such as *G. affinis*. The water temperature in Long Valley varied from 14.4 to 35.0 °C as recorded in different types of farm plots in 2010–2011 (Ma, 2012).

2.3. Pre-test handling of sample animals

Egg masses were immediately transported to the laboratory and transferred to acrylic aquaria filled with dechlorinated tap water (pH, 6.9–7.2; conductivity, $0.1467 \pm 5.5 \times 10^{-4}$ mS cm^{-1}). They were held under a photoperiod of 12 h light: 12 h dark at a relatively stable laboratory temperature of 25 °C (± 1 °C). At least three clutches of eggs were collected for each species. Hatchlings were subjected to periodic feeding with ground commercial fish flakes (TetraMin®, Tetra, Melle, Germany), and tadpoles were used in experiments when they reached free-swimming Gosner stage 25 (Gosner, 1960) (approximately 7–14 days). Tadpoles from different clutches were pooled together for subsequent experiments to minimize parental effects on experiments.

Gambusia affinis were kept in aerated glass aquaria under the same conditions as amphibians and were acclimated to laboratory conditions for at least two days before any experimentation. Fish were fed a mixture of commercial fish flakes (TetraMin®, Tetra, Melle, Germany) and frozen blood worms (Diptera: Chironomidae).

2.4. Determination of median lethal temperature (LT50)

Median lethal temperatures were determined for the three amphibian species and *G. affinis* following the chronic lethal temperature methodology (Alcorn, 1976; Fields et al., 1987; Shafland and Pestrak, 1982). Experimental animals were subjected to a gradual increase or decrease of temperature from the control temperature (stable laboratory temperature), and the corresponding cumulative mortality at each temperature point was recorded to calculate the temperature lethal to 50% of study animals (LT50).

Animals were exposed to three treatments, increasing temperature, decreasing temperature, and control, and each treatment was replicated three times. A 5-L glass jar, containing 4.5 L of dechlorinated tap water, represented an experimental unit and each contained 20 tadpoles or 20 mosquitofish. Three large water baths were set up with an aquarium chiller (Titan 150, Aqua Medic, Bissendorf, Germany) and/or a heating immersion circulator (Julabo ED, Julabo Labortechnik, Seelbach, Germany) to provide the temperature treatments. Initial temperatures of the three water baths were set at 25 °C. Temperature in a water bath was increased or decreased in a stepwise manner at a rate of 1 °C every 12 h. The remaining water bath served as the control with the temperature being kept at 25 °C for the entire experimental period. A photoperiod of 12 h light: 12 h dark was maintained for all water baths throughout. All subject animals were subjected to periodic feeding with ground commercial fish flakes (TetraMin®, Tetra, Melle, Germany) once every two days. About 50% of the water was changed with pre-heated/pre-cooled dechlorinated water on every non-feeding day to maintain good water quality. Cumulative mortality was recorded for each experimental unit every 12 h and dead animals were removed once observed. Death was defined as a lack of body movement and apparent heart beat. The experiment was terminated once all animals in the two treatment groups other than the control had perished, or after when the decreasing temperature treatment reached 4 °C, which was the lowest temperature that the setup could achieve.

2.5. Physiological responses at different temperatures

Polypedates megacephalus was used as the focal species to examine physiological responses of native amphibian larvae along a thermal gradient. Its construction of foam nest meant that the eggs would not be previously submerged in any water body, rendering them free from aquatic contaminants. Its relatively high abundance also made it a favourable subject animal. Twelve tadpoles each were placed in 5-L glass containers filled with 4.5 L of dechlorinated water. Tadpoles were maintained at relatively low densities to ensure that results were attributable to the factors of interest rather than to density-dependent effects (Browne et al., 2003). Tadpoles of *P. megacephalus* were exposed to eight temperatures within their thermal tolerance range (13, 16, 19, 22, 25, 28, 31 and 34 °C) for 10 days. Each temperature treatment was replicated four times.

Water baths with temperature controlled by an aquarium chiller (Titan 150, Aqua Medic, Bissendorf, Germany) and/or a heating immersion circulator (Julabo ED, Julabo Labortechnik, Seelbach, Germany) provided temperature treatments. Tadpoles were acclimated at the experimental temperature for 2 d before the start of the experiment. To do this, a gradual temperature increase or decrease of 1 °C/h from ambient temperature was employed until the desired experimental temperature was reached (Li et al., 2015). Water was changed at the end of the acclimation period. For each temperature, four additional replicates were acclimated concurrently and tadpoles were anaesthetised with MS-222 at the end of the acclimation period (day 0) for initial body length and body mass measurements.

During the exposure, tadpoles were subjected to period feeding with commercial fish food (Sera® Micron, Sera, Heinsberg, Germany) every day and the water was changed every four days. By the end of the exposure (day 10), four randomly-selected tadpoles were taken from each replicate for measurement of oxygen consumption rate. Remaining tadpoles were euthanized with an overdose of MS-222, weighed, and photographed for body length measurement. Tadpole samples were then frozen immediately in liquid nitrogen and stored at −80 °C for protein expression analysis.

2.5.1. Growth

Tadpoles were weighed to 0.1 mg at day 0 and day 10 using an electronic balance (Shimadzu AUW220D, Shimadzu Corporation, Japan). Body lengths of tadpoles were measured from photographs taken at day 0 and day 10 using image analysis software (ImageJ, version 1.47). The overall mean body mass and body length of the four replicates at day 0 were used as the initial mass and body length. Changes in body mass and body length of individual tadpoles at day 10 were calculated by subtracting the mean initial values from the corresponding values at day 10 for each replicate.

2.5.2. Oxygen consumption rate (OCR)

Oxygen consumption rate for *P. megacephalus* was measured using a dissolved oxygen measurement system (Strathkelvin 928, Strathkelvin Instruments, Glasgow, UK). Four tadpoles were randomly selected from each experimental unit and transferred to four individual airtight test chambers. Chambers were completely filled with 3 mL autoclaved milli-Q water and were placed in a water bath at the exposure temperature to maintain constant temperature throughout. The setup was recalibrated at each experimental temperature and all chambers were stirred with a magnetic stirrer to ensure accurate measurements. Animals were acclimated in chambers for 15 min before measurements began. The level of dissolved oxygen in each chamber was measured continuously for 30 min and slope of dissolved oxygen level against time was taken as the gross OCR of the tadpole. After measurement, tadpoles were anaesthetised with MS-222, weighed, and photographed for body length measurement. Gross OCR was normalized to µL O_2 per mg body mass per hour by the volume of water, duration of measurement, and mass of the tadpoles, i.e. $(O_{2\ \mathrm{initial}} - O_{2\ \mathrm{final}})/(\text{volume of water} \times \text{experimental duration} \times \text{body mass})$.

2.5.3. Expression of heat shock protein 70 (HSP70)

Three frozen post-exposure tadpoles from the same replicate were pooled together and homogenized in cold lysis buffer (pH 8.0; 50 mM Tris-HCl, 150 mM NaCl, 1% [*v*/*v*] Triton X-100, 10 mM DTT, 1 × Protease Inhibitor Cocktail [Roche Molecular Biochemicals, USA]). Lysates were centrifuged at 10,000 ×*g* at 4 °C for 15 min to remove debris. The total amount of proteins present in each sample was determined by the Bradford assay.

For each sample, 5 µg of protein was separated by electrophoresis on SDS-PAGE gels (Criterion TGX, Any kD, 18 well, 30 µL, Bio-Rad, USA). Separated proteins were transferred onto nitrocellulose membranes and stained with Ponceau S solution to confirm equal protein loading. Membranes were washed with 5% [*w/v*] skim milk in TBS-T (20 mM Tris-Hcl, pH 7.4, 154 mM NaCl, 0.1% [*v/v*] Tween-20) to block non-specific binding sites. Membranes were then incubated at 4 °C overnight with 1:4000 monoclonal mouse primary antibody (BioReagents, USA), followed by 1-h incubation at room temperature with 1:2000 anti-mouse IgG, secondary antibody (Zymed Laboratories, USA). ECL Western Blotting Detection Kits (Amersham Biosciences, UK) and light-sensitive Fuji X-ray films were used to detect antigen-antibody complexes on the blots. The amount of HSP70 present in the sample was determined by quantifying the gel band density using a densitometer (GS-800, Bio-Rad, USA) and expressed as relative optical density^{-1}. All samples were measured in duplicate.

2.6. Data analysis

Median lethal temperature (LT50) for each target species was calculated using a sigmoidal dose-response (variable slope) non-linear regression. Comparisons were made by checking for overlap between 95% confidence intervals; groups were deemed significantly different if there was no overlap. All analyses were conducted using the software GraphPad Prism 5.00 (GraphPad Software, CA).

Relationships between temperature and growth (in terms of changes in body length and mass) were determined by fitting a nonlinear model following Briere et al. (1999). The reciprocal of HSP70 expression level (HSP70′) was taken to flip the curve such that it would open downward and that the same model with temperature could be fitted. The equation of the model is given by:

$$y = aT(T - T_0)\sqrt{(T_L - T)}$$

where y = changes in body length or changes in mass or HSP70′, and the three parameters are a (a scale parameter), T_0 (lower thermal limit) and T_{L} (upper thermal limit) y is a positive function of temperature T in degree Centigrade.

From the models, the optimal temperature for *P. megacephalus*, i.e. the temperature at which performance was best, i.e. the highest growth rate or the least sign of stress, was then computed using the equation (Briere et al., 1999):

$$[4T_L + 3T_0 + \sqrt{(16T_L{}^2 + 9T_0{}^2 + 16T_L T_0)}]/10$$

A linear regression model was fitted to investigate the relationship between temperature and OCR (Clarke and Johnston, 1999; Feder, 1982). All models were fitted using R programming language (v.3.5.2; R Core Team 2018)

3. Results

3.1. Median lethal temperature (LT50)

Target species showed different levels of tolerance to high and low temperatures (Fig. 1). *Gambusia affinis* had the largest overall range of thermal tolerance (1.0–38.2 °C), followed by *D. melanostictus* (5.9–41.0 °C), *M. pulchra* (6.1–40.5 °C), and *P. megacephalus* (5.5–39.5 °C). *Duttaphrynus melanostictus* was the most tolerant of high temperature, and *P. megacephalus* and *G. affinis* were least tolerant of high temperature. Invasive *G. affinis* was the most tolerant of cold temperatures. Control mortality was less than 20% for all species (Fig. 2).

3.2. Physiological responses at different temperatures

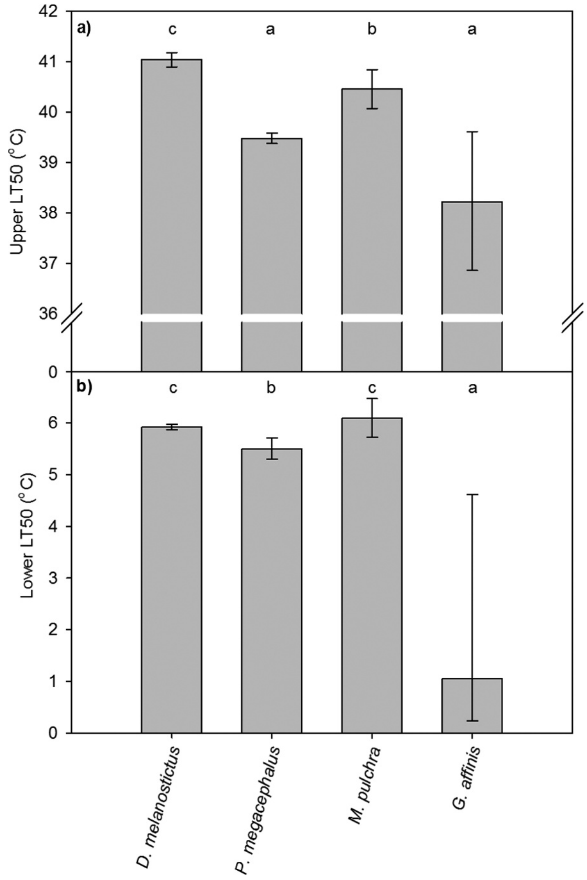

Fig. 1. a) Upper and b) lower median lethal temperatures (LT50) of the three amphibian species and the invasive fish *Gambusia affinis* from Hong Kong. Error bars represent 95% confidence intervals. Different letters above the bars indicate significant differences between the groups as determined by non-overlapping 95% confidence intervals.

Parameter estimates from the nonlinear regressions are summarized in Table 1. Plots of the fitted curves are shown in Fig. 1a–c. Optimal temperatures of *P. megacephalus* for different responses varied, with growth being favored at a slightly higher temperature (21.3–21.6 °C), while it appeared to be the least stressed at 18.0 °C in terms of HSP70 expression.

A significant linear relationship between temperature and OCR was observed in the larvae of *P. megacephalus* ($F_{1,30}$ = 203.94, p < 0.001; Fig. 1d). The relationship is described by the equation: OCR = 0.1238 × Temperature – 0.8515 and the model has a rather high explanatory power with a R^2 value of 0.872.

4. Discussion

4.1. LT50 and its relation to the environment

Use of chronic lethal methods allowed for estimation of the thermal limits of the target species independent of the pre-test acclimation temperature (Beitinger et al., 2000). The native range of *G. affinis* extends northwards from the Gulf Coast of northeastern Mexico up to 40°N latitude in the United States, and this fish is essentially a temperate species (Krumholz, 1944). Given that *G. affinis* has been established in Hong Kong since the 1940s (Dudgeon and Corlett, 2004) and is capable of producing 3–4 successive generations in a single breeding season, 200 generations may have been produced to date and possibly more given warmer conditions in recent decades (Pyke, 2005). One would expect this species to have adapted to the warmer South China climate after so many generations, however, the current thermal tolerance data suggest that this may not be the case. Relative to the local

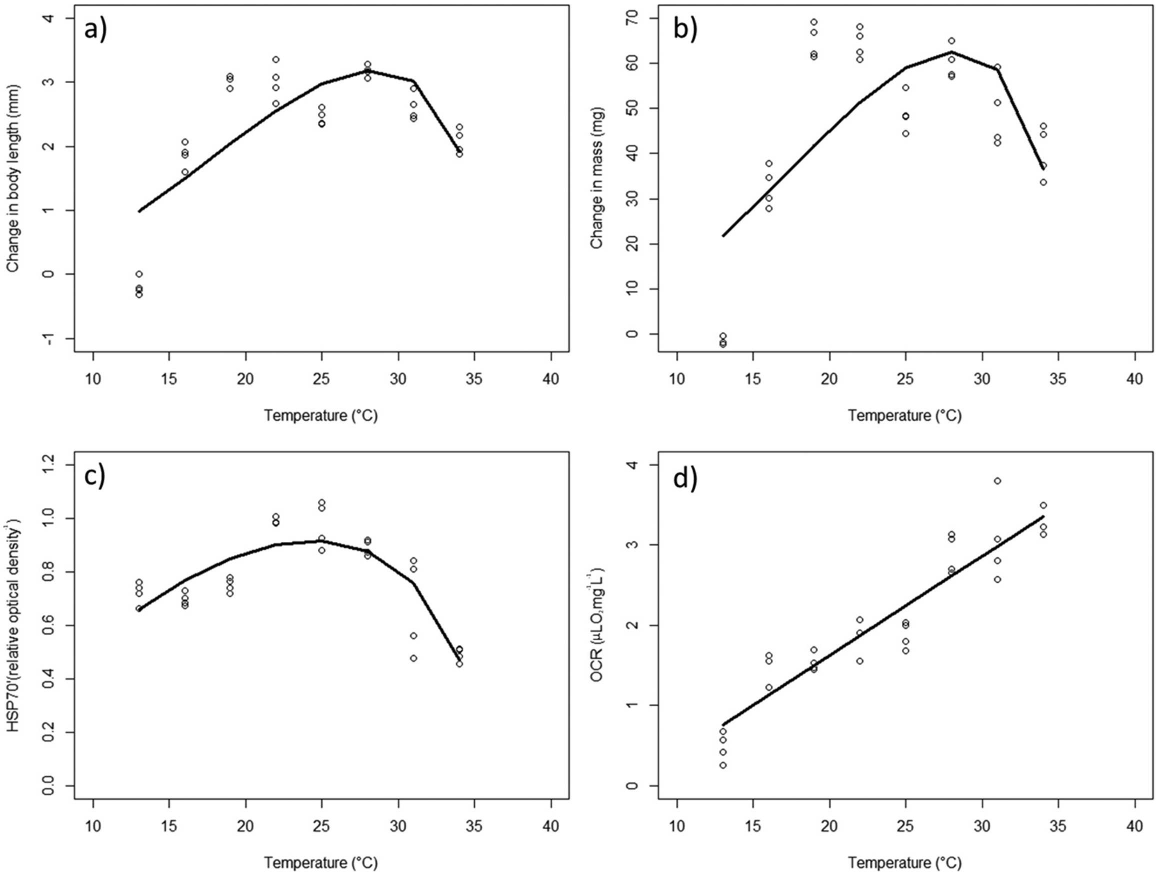

Fig. 2. Relationships between temperature and a) change in body length (mm); b) change in mass (mg); c) reciprocal of heat shock protein 70 expression (HSP70′; relative optical density $^{-1}$); and d) oxygen consumption rate (OCR; μgO$_2$ mg^{-1} L^{-1}) for *P. megacephalus*. Solid lines represent the regression models.

Table 1

Parameter estimates and their standard errors for the nonlinear models for changes in body length (in mm; BL), changes in body mass (in mg; M) and reciprocal of heat shock protein 70 expression (HSP70′). Optimal temperatures (in °C; T_{opt}) were estimated based on the models.

	a	SE	T_L	SE	T_0	SE	T_{opt}
BL	1.76×10^{-3}	3.88×10^{-4}	35.1	0.53	3.76	3.83	21.6
M	3.21×10^{-2}	8.53×10^{-3}	35.1	0.59	1.91	5.08	21.3
HSP70′	5.61×10^{-5}	5.88×10^{-5}	35.3	0.42	−178.0	207.0	18.0

amphibian species tested whose native distributions fall primarily within the tropics, *G. affinis* was not as tolerant of high temperature as most amphibians and was much more tolerant of cold temperatures. It should be noted, though, that the lower LT50 value of *G. affinis* was estimated by extrapolating the data to beyond the minimum test temperature, as the lowest temperature that the water bath could reach was 4 °C, at which average mortality of *G. affinis* was just 28%. Nevertheless, the high tolerance of *G. affinis* to low temperature was evident from this experiment.

The threat posed by *G affinis* on amphibian species is evident and has been recorded in different ecosystems across the globe. In California, the California newt (*Taricha torosa*) was absent from streams where they had previously been recorded as a result of predation by *G. affinis* (Gamradt and Kats, 1996). In Australia, the presence of *G. holbrooki* (a similar species to *G. affinis*, also commonly known as the mosquitofish) drove an endangered local frog species *Litoria aurea* to use suboptimal ephemeral habitats which may have been responsible for their declines (Hamer et al., 2002). *G. affinis* has also been proven to have an appetite for local amphibians in Hong Kong (Karraker et al., 2010; Lau, 1998). Although *G. affinis* had a lower tolerance to high temperature compared to two amphibian species, such a difference does not confer any apparent advantages to the amphibians. The upper thermal limit of *G. affinis* was higher than the mean summer water temperature in Long Valley of about 30 °C and the highest record of 34.3 °C (Ma, unpublished data). On the contrary, *G. affinis* may benefit from high summer temperatures. A preference for high temperatures of around 31–35 °C has been observed for *G. affinis* (Pyke, 2005). Other research (Vondracek et al., 1988) has also shown dramatic increases in fitness in *G. affinis* with temperature increases from 20 to 30 °C: mean age at first reproduction greatly shortened from more than six months to less than two months, coupled with the production of larger offspring with larger brood size, and such fitness parameters also increased with food availability. In other words, *G. affinis* is strongly favoured by the high temperature and abundance of amphibian larvae as prey (Karraker et al., 2010) during the breeding season, and therefore may chronically impact the wetland amphibian community.

Lower LT50 values for the three amphibian species ranged from 5.5 to 6.0 °C. These values are lower than the temperature that these species normally encounter, especially for *M. pulchra* and *P. megacephalus,* which usually start breeding in March, the earliest time at which temperatures begin to warm. Breeding as early as February, *D. melanostictus* may be exposed in some years to such low temperatures, but it is unlikely that this species would become active under such extremes. Over the period of 2010–2014, there have been a total of six days with air temperatures of 6 °C or lower and 36 days with temperatures below 10 °C recorded in February from the monitoring station nearest to Long Valley, and the lowest record was 1.3 °C (Hong Kong Observatory; http://hko.gov.hk). That said, water temperatures generally remain a few degrees above that of the air temperature during the cooler dry season.

4.2. Temperature-dependent growth

Growth (changes in body length and mass) of *P. megacephalus* was temperature-dependent with the optimal temperature occurring between 21 and 22 °C. Changes in both body length and mass were negative at 13 °C, which is believed to be a result of the observed reduction in activity and feeding. Even though the lower thermal limit of *P. megacephalus* was 5.5 °C, the results from the growth performance analysis suggested that at 13 °C or below, tadpoles would not be able to survive over the long-term as they were using up their energy reserves.

Larval growth rate is an important parameter in the life history of amphibians. According to the predictive models for metamorphic timing (Rose, 2005), tadpoles must attain a species-specific minimum size to initiate metamorphosis and there also exists a maximum size at which tadpoles can no longer postpone metamorphosis. Tadpoles may undergo metamorphosis at any size between the minimum and maximum size depending on the environmental conditions. Under suboptimal conditions, tadpoles may metamorphose earlier (Brunelli et al., 2009) or later at a smaller size (Cauble and Wagner, 2005), or may take a longer time to reach a particular size at metamorphosis (Peles, 2013).

A recent study on the North African green frog (*Pelophylax saharicus*) showed that the temperature at which maximum growth (in terms of weight gain) was recorded also fell within the range of maximum rate of metamorphosis (Bellakhal et al., 2014). By growing at a faster rate, amphibians spend less time as larvae, which is a more vulnerable stage of their life cycle (Wilbur, 1980). Amphibians that metamorphose more quickly and at a larger size are believed to not only be more resistant to predation, both before and after moving to land, but also exhibit higher fitness by reaching sexual maturity more quickly and at a larger size (Harris, 1999).

4.3. Thermal effects on metabolic rate of P. megacephalus

Oxygen consumption rate is often used as a measure of metabolism (Marshall and Gordon, 1980). In the current study, the regression model explains 87.2% of the variance in OCR of *P. megacephalus* by temperature, meaning that temperature is the predominant factor that affects OCR. OCR measurements alone are not indicative of the animals' aerobic capacity, as the determination of aerobic scope requires measurements of maximal metabolic rate as well (Clarke and Pörtner, 2010). Aerobic scope is often limited by the restricted capacity of the animal's circulatory system to supply oxygen at low temperatures, and the insufficient level of dissolved oxygen and increased metabolic demand at high temperatures (Pörtner, 2010). Nevertheless, the measurement of OCR provides us with an understanding of the relationship between basal metabolic rate and temperature.

4.4. Molecular physiological responses to temperature changes

Heat shock proteins can be up-regulated by the presence of different stressors, including thermal stress (Robert, 2003). Heat shock proteins are known as molecular chaperones which interact with other proteins as a protection mechanism (Feder and Hofmann, 1999). Increased expression levels of HSP70 were observed at both low and high temperatures as predicted by the oxygen- and capacity-limited model (Pörtner, 2010), which suggests that ectotherms increase their reliance on anaerobic metabolism and induce heat-shock response as temperature falls or rises to a certain extent beyond the optimal temperature. The elevated expression of HSP70 was indicative of protein damage, and thus an increase in energy demand for cellular repairing and protection mechanism.

4.5. Optimal temperature in relation to environmental temperature

Optimal temperatures for *P. megacephalus* ranged from 18.0 to 21.6 °C based on its growth and expression of heat shock protein. This suggests that overall optimal performance of larval *P. megacephalus* is likely to fall within that temperature range. In other words, despite having an upper thermal limit as high as 39.5 °C, larval *P. megacephalus* are likely to be stressed at least during part of the summer season with wetland water temperatures averaging 29–32 °C, and to above 34 °C from June to August (Ma, unpublished data). Although some species have shown increased thermal tolerance with developmental stage, a reduction in thermal tolerance has also been observed during metamorphosis (Cupp, 1980). This could mean a particularly challenging time for individuals that metamorphose in late summer when temperatures are warmest. Surviving beyond an optimal thermal window also means a reduced aerobic scope for activities beyond basal metabolism, which could render tadpoles more susceptible to other stressors (Farrell et al., 2008). For instance, temperature-dependent toxicity is commonly observed in aquatic ectotherms (Lau et al., 2014) and decreases in chemical tolerance with increasing temperature have been demonstrated in the same three amphibian species of the current study (Lau et al., 2015).

The latest report by the Intergovernmental Panel on Climate Change (IPCC Intergovernmental Panel on Climate Change et al., 2013) predicts median temperature rises of 1.5–2 °C by 2046–2065, and 3–4 °C by 2081–2100 in the worst case scenario, using temperatures between 1986 and 2005 as a baseline for comparison. Even though a difference of less than 2 °C in upper LT50 across the species tested may seem small, such a small difference may determine which species will persist under the changing climate.

5. Conclusions

Slight, but statistically significant differences in thermal tolerance have been observed among larvae of the three lowland amphibian species. The range of variation of their lower and upper tolerance limits was less than 1 °C and less than 2 °C, respectively. Although *G. affinis* is not as tolerant of high temperature as some of the amphibian species, it remains an important threat to lowland amphibian communities in South China as it has a thermal preference similar to local summer temperatures.

Investigation of the thermal physiology of *P. megacephalus* revealed that its optimal temperatures for physiological performance parameters fall within a range of 18.0–21.6 °C, which signifies that *P. megacephalus*, and possibly other amphibian species that share similar habitats and life history traits, are already under thermal stress at least during part of their larval stage. This could mean a reduction in their capacity to deal with other stressors. Moreover, *G. affinis* already presents a threat to amphibians in South China and this threat could potentially be exacerbated in the face of global climate change. As pollution and invasive predators are two of the most important threats to amphibians (IUCN, 2008), more in-depth studies on their effects on amphibians under different thermal scenarios are warranted.

Acknowledgements

This work was funded by the Research Grants Council, University Grants Committee, Hong Kong through a General Research Fund (Project No. HKU 703511P). Edward Lau was partially supported by the University of Hong Kong via a postgraduate studentship. The authors thank the anonymous reviewers for their helpful comments, and L. Ng and H. Leung for their technical support. The authors declare that there is no conflict of interest for the present study.

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