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ORIGINAL PAPER

Ontogeny of hypoxic modulation of cardiac performance and its allometry in the African clawed frog *Xenopus laevis*

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Abstract The ontogeny of cardiac hypoxic responses, and how such responses may be modified by rearing environment, are poorly understood in amphibians. In this study, cardiac performance was investigated in *Xenopus laevis* from 2 to 25 days post-fertilization (dpf). Larvae were reared under either normoxia or moderate hypoxia ($PO_2 = 110$ mmHg), and each population was assessed in both normoxia and acute hypoxia. Heart rate (f_H) of normoxic-reared larvae exhibited an early increase from 77 ± 1 beats min^{-1} at 2 dpf to 153 ± 1 beats min^{-1} at 4 dpf, followed by gradual decreases to 123 ± 3 beats min^{-1} at 25 dpf. Stroke volume (SV), 6 ± 1 nl, and cardiac output (CO), 0.8 ± 0.1 µl min^{-1}, at 5 dpf both increased by more than 40-fold to 25 dpf with rapid larval growth (~30-fold increase in body mass). When exposed to acute hypoxia, normoxic-reared larvae increased f_H and CO between 5 and 25 dpf. Increased SV in acute hypoxia, produced by increased end-diastolic volume (EDV), only occurred before 10 dpf. Hypoxic-reared larvae showed decreased acute hypoxic responses of EDV, SV and CO at 7 and 10 dpf. Over the period of 2–25 dpf, cardiac scaling with mass showed scaling coefficients of −0.04 (f_H), 1.23 (SV) and 1.19 (CO), contrary to the cardiac scaling relationships described in birds and mammals. In addition, f_H scaling in hypoxic-reared larvae was altered to a shallower slope of −0.01. Collectively, these results indicate that acute cardiac hypoxic responses develop before 5 dpf. Chronic hypoxia at a moderate level can not only modulate this cardiac reflex, but also changes cardiac scaling relationship with mass.

Keywords Cardiac function · Scaling · Ontogeny · Hypoxia · *Xenopus laevis* · Amphibians

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Introduction

The development of cardiovascular function in amphibians has received considerable attention, especially in larval anurans (Burggren and Doyle 1986; Burggren et al. 1992; Hou and Burggren 1995a, b; Fritsche and Burggren 1996; McKenzie and Taylor 1996; Tang and Rovainen 1996; Jacobsson and Fritsche 1998; Territo and Altimiras 1998; Warburton and Fritsche 2000; Mckean et al. 2002). In *Xenopus laevis*, the developing heart starts beating as early as 2 days post-fertilization (dpf) when hatching is about to occur (Nieuwkoop and Faber 1967). Within 3 days after the first heartbeat, the larval heart has progressed through looping, atrial septation and development of atrioventricular valves (Nieuwkoop and Faber 1967; Kolker et al. 2000). Along with rapid cardiac development, actual cardiovascular performance also undergoes rapid changes during larval stages. Mean arterial pressure and stroke volume (SV) in *X. laevis* both increase sharply in early development, closely associated with increasing body mass (Burggren 1995; Hou and Burggren 1995b). Unlike SV and arterial pressure, the ontogeny of heart rate (f_H) is more variable and without a consistent pattern (Burggren 1995).

In *X. laevis* and *Lithobates catesbeianus* (formerly *Rana catesbeiana*), f_H increases initially after the onset of heartbeat, followed by significant decreases during later development (Burggren and Doyle 1986; Hou and Burggren 1995a; Territo and Altimiras 1998). On the other hand, f_H of *Pseudis paradoxsus* is relatively less affected by developmental stage (Burggren et al. 1992).

Similar to normoxic cardiac performance, cardiac responses to acute hypoxic exposure also vary interspecifically in amphibians. In early larval *X. laevis* (before 15 dpf), hypoxia depresses cardiac function by directly inhibiting cardiac muscle, which leads to a decrease in cardiac output (CO) (Orlando and Pinder 1995; Fritsche and Burggren 1996). In older larvae, however, cardiac reflexes appear and respond to acute hypoxia by decreasing f_H and increasing SV, yielding no net change in CO (Fritsche and Burggren 1996). In contrast, neither hypoxia nor hyperoxia affect f_H in larval *L. catesbeianus* examined (Burggren and Doyle 1986).

Although acute hypoxic cardiac responses have been characterized in anuran larvae, we know far less about how *chronic* hypoxia during early development may induce developmental plasticity in the form of adjustments in early cardiac performance. In one of the few such studies, in larval *X. laevis* neither f_H nor CO in normoxia was affected by a chronic exposure to hypoxia (PO_2 of 75 mmHg) (Territo and Altimiras 1998). Similar findings are evident for larvae of the salamander *Ambystoma tigrinum* (McKean et al. 2002). In zebrafish, chronic exposure to a PO_2 of ~75 mmHg increases normoxic f_H and CO (Jacob et al. 2002). In contrast, a more severe chronic hypoxia (PO_2 ~30 mmHg) attenuates not only f_H, but also overall development (Bagatto 2005).

Compounding analyses of hypoxic effects on cardiac performance are potential scaling effects, given that amphibian larvae concurrently grow enormously in body mass even as they go through profound developmental changes. Body mass is a powerful factor affecting the rate of biological processes, including cardiac performance (West et al. 1997; Dewey et al. 2008). The cardiac scaling relationship with mass can be described by the equation $Y = aM^b$, where Y is cardiac performance (which could include f_H, SV and CO), a is the scaling constant, M is body mass and b is the scaling exponent. As predicted by the analytic model based on the mammalian cardiovascular system, f_H, SV and CO typically scale with mass to a b of −0.25, 1 and 0.75, respectively (West et al. 1997). However, existing data on ectothermic vertebrates are limited and show conflicting mass and f_H relationships, with b ranging from −0.12 to 0.25 (Hou and Burggren 1995a; Mirkovic and Rombough 1998; Clark and Farrell 2011), in contrast to the predicted value of b of −0.25. In developing rainbow trout (*Oncorhynchus mykiss*), the increases in SV and CO are greater than body mass increase, with b of 1.54 (SV) and 1.78 (CO), respectively (Mirkovic and Rombough 1998).

Although the effect of body mass on cardiac performance remains variable, the degree to which the cardiac system is affected by acute or chronic change in the ambient PO_2 may also be size dependent. In fish, temperature and pH have been shown to affect metabolic scaling relationships (Ohlberger et al. 2007; Killen et al. 2010; Vaca and White 2010), but the effect of oxygen environment on cardiac allometry has not been studied in ectothermic vertebrates. In addition, no study has characterized early development of acute cardiac responses as influenced by development in chronic hypoxia in a rapidly developing amphibian species, *X. laevis*. Thus, the aims of this study were to explore the developmental plasticity of the ontogeny of acute cardiac responses in *X. laevis* reared in normoxic and chronic hypoxic conditions. We hypothesized that the early cardiac performance measured in normoxia and acute hypoxia will be altered by chronic hypoxia in a body mass-dependent fashion.

Materials and methods

Animals

Adult *X. laevis* used for breeding were obtained from XENOPUS 1 (Dexter, MI, USA). Frogs were kept under a 14 h:10 h light:dark cycle at 22 ± 2 °C and fed daily with commercial frog food. To induce breeding, human chorionic gonadotropin (hCG, Sigma-Aldrich Corp., St. Louis, MO) was injected into the dorsal lymph sac with a 1 ml syringe and a 26-gauge needle, following standard breeding protocols. A total of three breeding pairs, which produced four clutches of fertilized eggs, were used in the study. All procedures were approved by UNT Institutional Animal Use and Care Committee.

Chronic exposure to hypoxia

Fertilized eggs were separated into two groups of ~100 eggs within 3-h post fertilization. Each group was placed in either a covered normoxic tank (PO_2 of 150 mmHg) or hypoxic (PO_2 of 110 mmHg) tank at a density of ~5 individuals·l^{-1}. A PO_2 of 110 mmHg was selected because pilot experiments with larval *Xenopus* indicated that this level of hypoxia was severe enough to elicit physiological responses in larvae, but not so severe as to prevent normal rates of development (Pan and Burggren 2010). Our goal was to examine the effects of a moderate level of hypoxia and the resultant effects that might emerge, rather than inducing the potentially teratogenic effects of more severe hypoxia.

Incubation tanks were kept in a water bath maintained at 22 ± 1 °C. Larvae were fed daily with powder made from frog food (XENOPUS 1, INC, Dexter, MI), with frequent water changes to prevent water fouling. Water in the normoxic tank was kept aerated by bubbling with room air, and hypoxic water was equilibrated with a gas mixture of N_2 and air to produce a PO_2 of 110 ± 5 mmHg. PO_2 in the gas mixture and water were measured with fiberoptic O_2 probes (Ocean Optic Inc., Dunedin, FL), which were connected to a multifrequency phase fluorometer (MFPF-100, TauTheta Instruments LLC, Boulder, CO). OOISensors software was used to obtain PO_2 readings. Both aerial and aquatic PO_2 in the hypoxic tank were kept at 110 ± 5 mmHg during the entire incubation period.

Larval restraint

Larvae at each stage were transferred to a water flow-through measurement chamber, which was modified from either a 16- or a 24-well cell culture plate (Falcon, BD Bioscience, San Jose, CA), depending on the size of larvae (see Pan and Burggren 2010, for details). The bottom of each water-filled well was filled with a layer of solidified 1.5 % agarose gel. A smaller, central space in which an unanaesthetized larva was placed was carved out of the agarose surface. The longitudinal size of the space varied with the size of larvae at each stage. A piece of nylon mesh was then put on the gel over the larva to gently confine it to the hollowed-out space. The holding chamber with larval *X. laevis* was then transferred to the stage of an inverted microscope (TS-100, Nikon).

At the beginning of each measurement, animals were placed in wells and received normoxic water for 30 min. After the first measurement in normoxic water, larvae were exposed to water with PO_2 of 110 mmHg and then a final exposure to 70 mmHg. A 10-min period was allowed for larvae to adjust at each O_2 level before measurements were made. Flowing water surrounding the larva in its agarose well was monitored using fiberoptic O_2 probes.

Cardiac measurements

f_H was measured from stage 35/36 (2 dpf—one stage after onset of heartbeat) to stage 53 (~25 dpf) in both normoxic and chronically hypoxic populations. f_H was measured by direct counting using an inverted microscope.

SV and CO were measured from stage 46 and 47 (~5 dpf), at which looping, valve and chamber formation and atrial septation are completed (Nieuwkoop and Faber 1967; Kolker et al. 2000; Warkman and Krieg 2007). To measure SV, the beating heart of the restrained larva was recorded with a video camera (Javelin Electronics, Japan) on an inverted microscope at a rate of 30 frames s^{-1}. A total magnification of 100× was used for larvae younger than 10 dpf, while 50× was used for larvae between 10 and 25 dpf. At least 10 cardiac cycles were recorded and used for calculation of SV in each animal. Calculation of ventricular volume followed the method used by Hou and Burggren (1995b) based on the assumption that the ventricle is spherical and the volume of the ventricular wall remains constant during contraction and relaxation. In brief, the frame containing the largest ventricle size was used to calculate end-diastolic volume, and the smallest ventricle size was used to calculate end-systolic volume. The ventricles in selected frames were analyzed using Image-Pro software to obtain the long and short radius of the heart. Ventricular volume was calculated by the formula, $V = 4/3\pi ab^2$, where a is the long radius and b is the short radius of the ventricle. SV was determined by calculating the difference of end-diastolic and end-systolic volumes. Animals were weighed after measurements of all cardiac variables.

Statistical analysis

The overall effects of age, acute and chronic hypoxia on f_H, SV and CO were analyzed using three-way analysis of variance (ANOVA). If statistical differences were seen, additional two-way repeated measures ANOVAs were performed, followed by Tukey's multiple comparison tests, to assess the differences between levels of acute and chronic hypoxia at each age or between age and acute hypoxia within each chronic treatment group. The effects of hypoxia on cardiac scaling coefficients were tested using analysis of covariance (ANCOVA). All values in the figures except for Fig. 5 are shown as mean ± 1 standard error of the mean.

Results

Ontogeny of cardiac performance in normoxia

To show ontogenetic patterns of cardiac performance, f_H, SV and CO are plotted against age in Figs. 1 and 2. f_H in larvae raised and measured in normoxia increased sharply and significantly ($P < 0.001$) from 77 ± 1 beats min^{-1} at 2 dpf to 107 ± 2 beats min^{-1} at 3 dpf, then peaked at 4 dpf at a rate of over 150 beats min^{-1} (Fig. 1). Normoxic f_H then remained constant for ~20 days before finally decreasing significantly to 123 ± 3 beats min^{-1} at 25 dpf (Fig. 1).

Normoxic SV increased dramatically with age ($P < 0.001$, Fig. 2a), from 30 ± 3 nl at 10 dpf to 340 ± 38 nl at 25 dpf (Fig. 2a). Larval CO showed a similar pattern, with a 10-fold increase from 4 ± 1 µl min^{-1} at 10 dpf to 41 ± 4 µl min^{-1} at 25 dpf (Fig. 2b). The sharp increase in

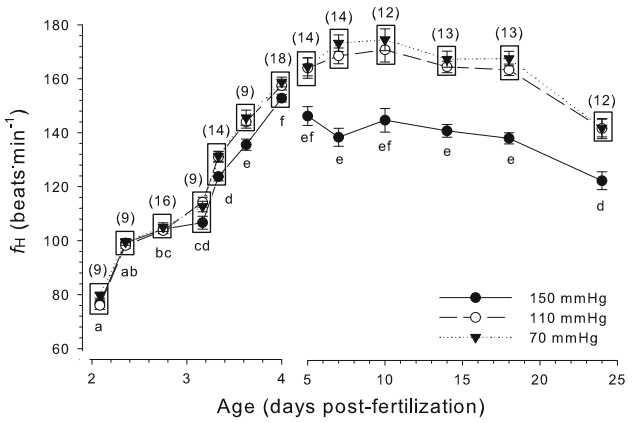

Fig. 1 Ontogeny of heart rate (f_H) as well as f_H response to acute hypoxic exposure (110 and 70 mmHg) in normoxic-reared larvae. Note the time scale breaks from days 4 to 5. *n* in *parentheses*. *Letters* show significant differences among age in normoxic f_H (*solid line*). *Boxes* enclose statistically identical means. Mean ± SE are plotted

SV and CO after 10 dpf was related to the ontogenetic changes in body mass, which increased from 11.4 ± 2.6 to 119.5 ± 42 mg between 10 and 25 dpf (Fig. 2c). In contrast to the sharp increases in SV and CO when plotted against age, mass-specific SV and CO showed no change after 10 dpf (Fig. 2d. e); that is, after the early increase, SV and CO per wet mass remained constant during later stages examined in this study.

Cardiac responses to acute hypoxia

Increased f_H in response to acute hypoxia was the most significant and consistent cardiac response in larval *X. laevis* during early development ($P < 0.001$, Fig. 1). This response began as early as stage 43 (~3 dpf), with heart rate increasing from 136 ± 2 in normoxia to 146 ± 3 beats min^{-1} at the lowest O_2 level tested (PO_2 of 70 mmHg) (Fig. 1). A tachycardia in response to acute hypoxia was evident at all stages up to 25 dpf, the last stage examined (Fig. 1). Both tested levels of acute hypoxic exposure (110 and 70 mmHg) caused an average increase of about 20 beats min^{-1} above normoxic f_H.

Owing to the sharp increase in SV and CO during early development (relative to), volume is plotted against O_2 level in Fig. 3 to show the increase in cardiac performance in response to acute hypoxia. Stroke volume increased in response to acute hypoxia only at 5, 7 and 10 dpf (Fig. 3a–c). Larvae at these three stages exhibited an average increase of ~8 nl in SV as PO_2 fell from normoxia to 70 mmHg. In addition, a greater SV during acute hypoxic exposure resulted from increased end-diastolic volume (EDV). End-systolic volume (ESV) was maintained at all stages and O_2 levels examined (Fig. 3).

Given the effect of acute hypoxia on f_H and SV, not surprisingly CO also increased in acute hypoxia at all measured stages (Fig. 3). The magnitude of increase in CO was the greatest at the first two stages, at which a greater than 75 % increase occurred during acute hypoxic exposure. The magnitude of the response was attenuated to an approximate 40 % increase between 10 and 18 dpf, before it reached the lowest value of only a 15 % increase in CO at 25 dpf. At 25 dpf, the effect of acute hypoxia was still significant ($P = 0.011$), but post hoc analysis failed to find significant differences between acute O_2 levels.

Effect of chronic hypoxic rearing on acute cardiac responses

Chronic exposure to hypoxia (PO_2 of 110 mmHg) did not affect the acute hypoxic response of f_H or ESV at any stage examined. In contrast, acute hypoxic responses in SV and CO were decreased at 7 dpf, and EDV, SV and CO were decreased at 10 dpf, ($P = 0.003$ for CO at 7 dpf, respectively, and $P < 0.001$ for others, Fig. 4). At 7 dpf, chronic exposure to hypoxia only attenuated the acute response in SV from 16 ± 1 to 11 ± 1 nl, and CO from 2.7 ± 0.2 to 1.8 ± 0.3 μl min^{-1}, measured at a PO_2 of 110 mmHg (Fig. 4a, c, e). The effect of chronic hypoxia on acute hypoxic cardiac responses was greater at 10 dpf, when SV and CO were decreased approximately by 30 % at all O_2 levels tested (Fig. 4b, d, f).

Effect of chronic hypoxia on cardiac allometry

In addition to affecting the absolute values of cardiac performance, chronic hypoxia also altered cardiac scaling with mass. To show cardiac scaling, cardiac performance is plotted against mass in Fig. 5. In normoxic-reared larvae, f_H measured at all PO_2s showed significant relationships with mass ($P < 0.001$) with a *b* of −0.04 (Table 1). In contrast, chronic exposure to hypoxia modified the pattern of f_H scaling relationship with body mass, resulting in *b* between −0.01 and −0.02 ($P < 0.001$, Fig. 5a,), and only f_H measured at a PO_2 of 75 mmHg showed a significant regression against mass (Table 1). Unlike f_H, the scaling of SV and CO measured at all PO_2s acutely was not affected by chronic rearing in hypoxia (Fig. 5b, c).

Discussion

Ontogeny of cardiac performance in normoxia

The developmental pattern and absolute values of f_H in early larval *Xenopus* in the current study are comparable to those reported in earlier studies (Burggren 1995; Hou and

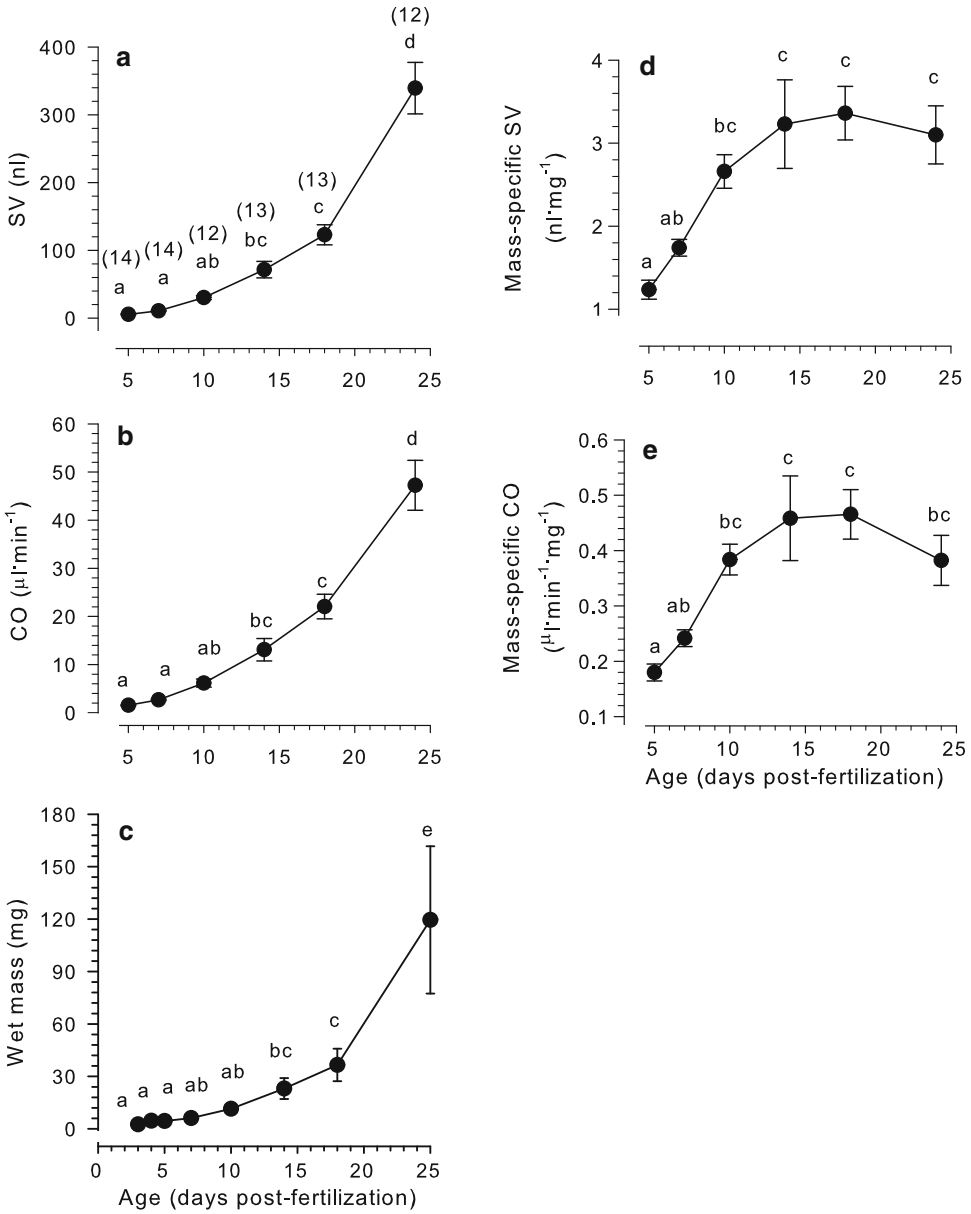

Fig. 2 Ontogeny of stroke volume (SV) (**a**), cardiac output (CO) (**b**), body mass (**c**), mass-specific SV (**d**) and CO (**e**) in normoxic larvae. *n* in *parentheses*. *Letters* show significant differences among age. Mean ± SE are plotted

Burggren 1995a; Orlando and Pinder 1995; Territo and Altimiras 1998). During this period, f_H increases from the onset of heartbeat to 5 dpf (Fig. 1). Later on, the sharp increase is followed by a period of gradual decrease between 5 and 21 dpf (Fig. 1). This pattern of change in f_H during development is typical of many lower vertebrates (Burggren and Warburton 1994; Burggren 1995, 2005). The early sharp increase in f_H, typical of many developing vertebrates (Burggren and Warburton 1994) may reflect a rapid cardiac development in *X. laevis*, which takes about 5 days for an adult-like heart to be developed (Nieuwkoop and Faber 1967; Kolker et al. 2000; Warkman and Krieg 2007). In addition, an early adrenergic tonus is present and peaks between 4 and 5 dpf (Jacobsson and Fritsche 1999), which coincides with the early peak in f_H observed in the present study.

The cause of the gradual decline in f_H after early organogenesis is still uncertain, and has been attributed to increasing body mass in larval *X. laevis* (Hou and Burggren 1995a; Orlando and Pinder 1995; Jacobsson and Fritsche 1999, Fig. 5 present study). Larvae increase in mass after 7 dpf, and a dramatic increase occurs especially after 14 dpf (Pan and Burggren 2010), when a drop in f_H appears (Fig. 1). Although a statistical significant relationship between f_H and mass exists, the regression model only explains 20 (Table 1) to 25 % (Hou and Burggren 1995a) of the variation in f_H. In addition, a *b* of ~0 in developing *X. laevis, L. catesbeianus, P. paradoxsus* and adult

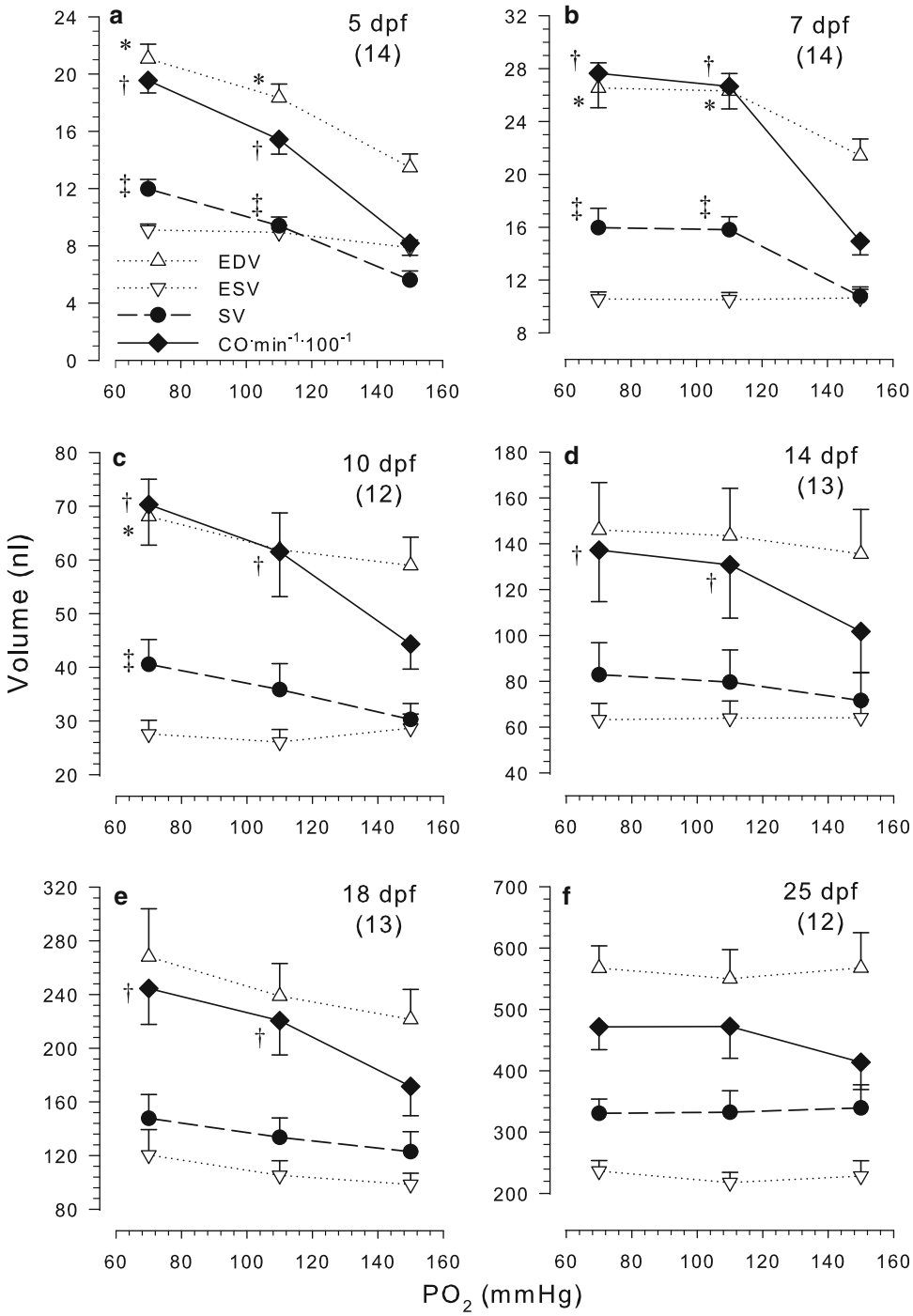

Fig. 3 End-diastolic volume (EDV *open triangle*), end-systolic volume (ESV *open inverted triangle*), stroke volume (SV *closed circle*) and cardiac output (CO *closed diamond*) responses to acute hypoxic exposures in normoxic larvae at six developmental stages. Note that *different scales* are used on *y* axis to show changes in volumes at each stage. *Asterisks*, *double daggers* and *daggers* show significant differences between normoxic and hypoxic EDV, SV and CO, respectively. *n* in *parentheses*

O. tshawytscha (Burggren and Doyle 1986; Hou and Burggren 1995a; Burggren et al. 1992; Clark and Farrell 2011) suggests that body mass is a relatively poor indicator for f_H in the above-mentioned ectothermic vertebrates. On the other hand, f_H of developing *O. mykiss* is dependent on, and increases with, body mass (Mirkovic and Rombough 1998). Overall, developmental changes in f_H of lower vertebrates are species-specific and cannot be predicted by the f_H—mass model in mammals (West et al. 1997). Possible reasons include the vastly different magnitude of changes in body mass among species, changes from hyperplastic to hypertrophic growth, as well as the differences in the development and modes of gas exchange. The early onset of air breathing in *X. laevis* (Pan and Burggren 2010), in conjunction with a sharp increase in SV (Fig. 2; Table 1), could allow larvae to access an oxygen-rich respiratory medium, thus ensuring proper oxygenation of the tissues with a relatively constant f_H during development.

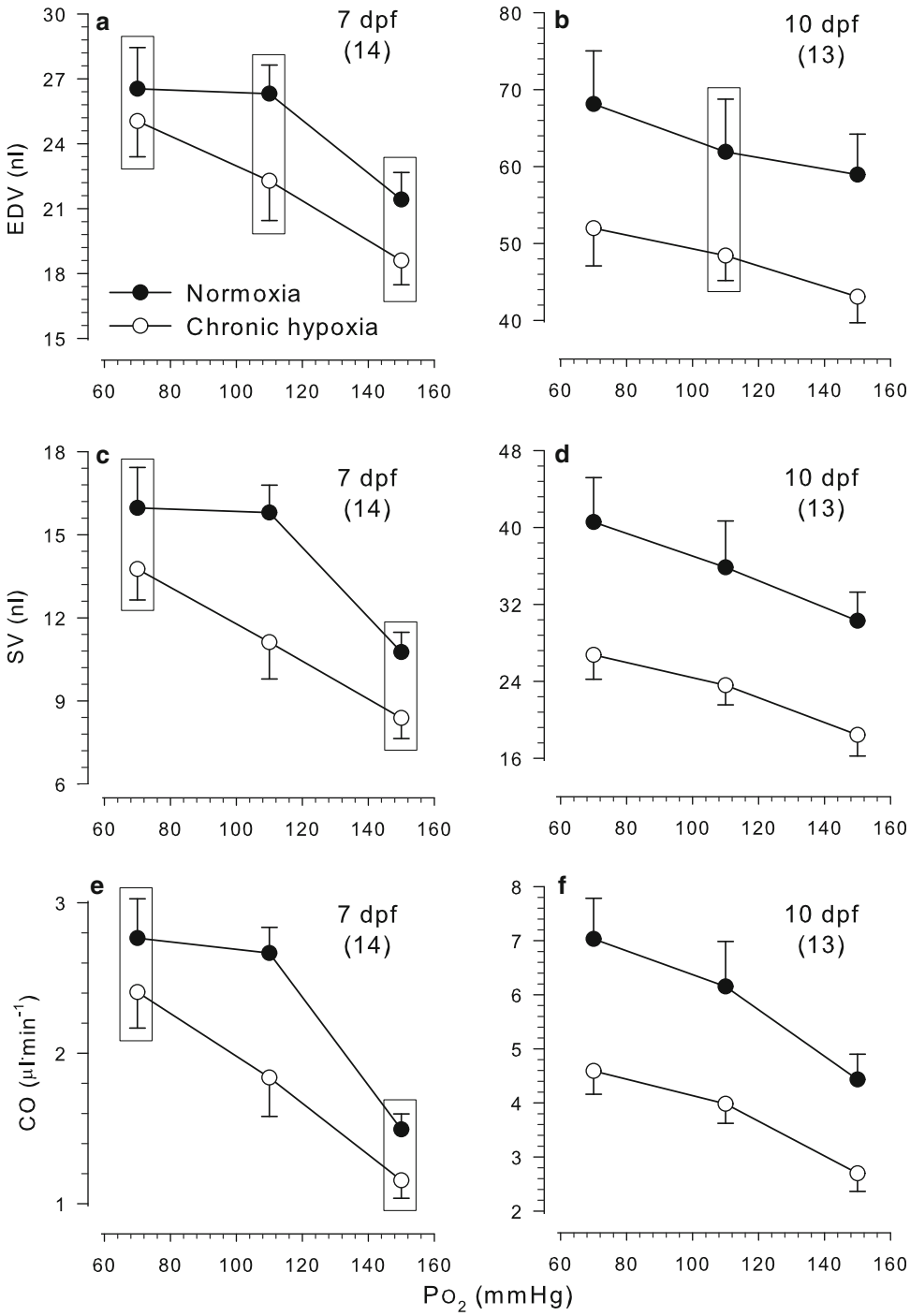

Fig. 4 Effects of chronic hypoxia (110 mmHg *open circles*) on the responses of end-diastolic volume (EDV) (**a**, **b**), stroke volume (SV) (**c**, **d**) and cardiac output (CO) (**e**, **f**) to acute hypoxic exposure at 7 (**a**, **c**, **e**) and 10 (**b**, **d**, **f**) dpf. Note that only selected stages at which cardiac variables are modified by chronic hypoxia are shown, and that *different scales* are used on *y* axes. *n* in *parentheses*. *Boxes* enclose statistically identical means. Mean ± SE are plotted

The developmental increases of both SV and CO were highly dependent on body mass (Figs. 2, 5), with *b* of 1.20 (Table 1), a value greater than the previously reported *b* of 0.96 for this species (Hou and Burggren 1995b). In their study, the whole larval period of *X. laevis* was investigated in contrast to the focus of ontogeny in the current study. A higher *b* during ontogeny shows a higher increase in SV and CO relative to body mass in early development. When expressing SV and CO on a mass-specific basis (Fig. 2c, d), ventricular volumes per body mass increased only between 5 and 10 dpf and then reached a constant value maintained at the later stages examined. This result indicates that during early development, the ratios of increase in SV and CO to mass are not constant in larval *X. laevis*. An early, sharp increase in SV and CO with body mass may be necessary for larvae to transport nutrients from the yolk to the rest of the body before active and efficient feeding starts.

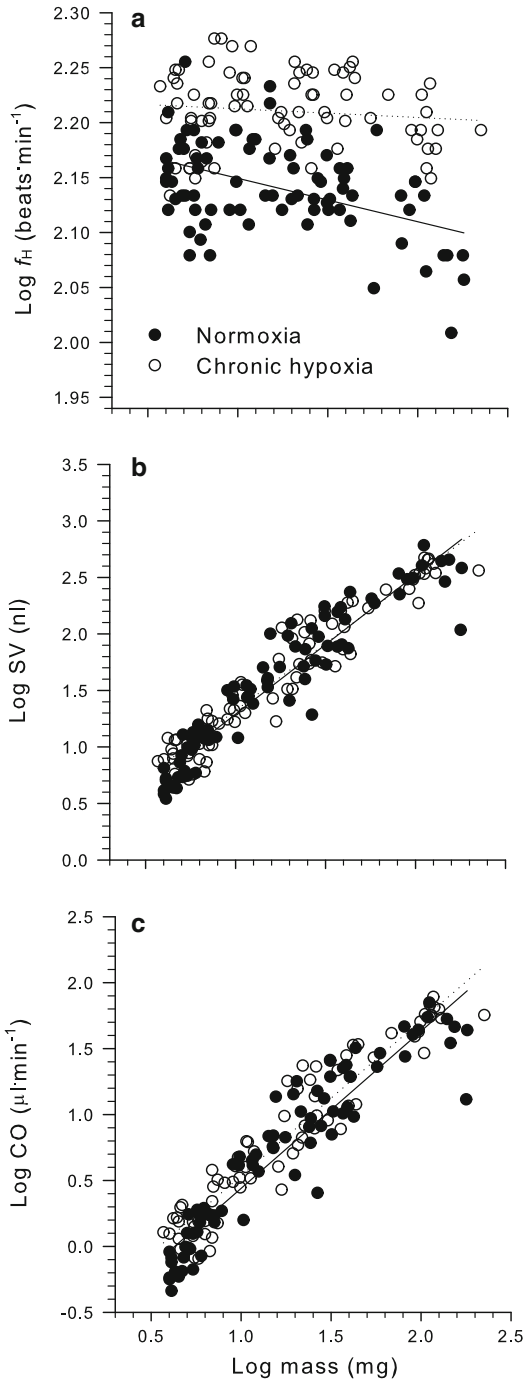

Fig. 5 Heart rate (f_H) (**a**), stroke volume (SV) (**b**) and cardiac output (CO) (**c**) scaling relationships with body mass in normoxic-reared larvae (*solid circle* and *solid line*) and hypoxic-reared larvae (*open circle* and *dotted line*). Note that values measured at rearing PO_2 (150 mmHg for normoxic and 110 mmHg for hypoxic larvae) are shown. Log values for f_H, SV and CO are given, see Table 1 for all other scaling relationships. Regression lines are described by **a** *solid line* $f_H = 2.19 \cdot \text{mass}^{-0.04}$ ($n = 78$, $r^2 = 0.20$, $P < 0.001$), *dotted line* $f_H = 2.22 \cdot \text{mass}^{-0.01}$ ($n = 77$, $r^2 = 0.01$, $P = 0.33$), **b** *solid line* $\text{SV} = 0.06 \cdot \text{mass}^{1.23}$ ($n = 78$, $r^2 = 0.89$, $P < 0.001$); *dotted line* $\text{SV} = 0.14 \cdot \text{mass}^{1.19}$ ($n = 77$, $r^2 = 0.92$, $P < 0.001$), **c** *solid line* $\text{CO} = -0.75 \cdot \text{mass}^{1.19}$ ($n = 78$, $r^2 = 0.88$, $P < 0.001$), *dotted line* $\text{CO} = -0.64 \cdot \text{mass}^{1.18}$ ($n = 77$, $r^2 = 0.91$, $P < 0.001$)

Ontogeny of cardiac responses to acute hypoxia

This is the first study reporting an early, strong tachycardia in response to acute hypoxia in *Xenopus*, appearing at 3 dpf and persisting to 25 dpf. Indeed, tachycardia is the major cardiac response observed over this developmental range (Fig. 1). The increase in f_H also contributed to increases in CO at all stages but 25 dpf (Fig. 3). Adrenergic innervation of the heart of *X. laevis* does not develop until metamorphosis (Kloberg and Fritsche 2002) and, consequently, the increase in f_H cannot be explained by changing sympathetic influence on the heart. However, even in the absence of autonomic innervation, circulating catecholamines produced by the chromaffin cells, known to be responsive to hypoxia (López-Barneo et al. 2001; Reid and Perry 2003; Perry et al. 2005), can bind to adrenergic receptors on the developing heart potentially producing positive inotropic and chronotropic responses. The early appearance of the cardiac adrenergic receptors has been reported in *Rana temporaria* at 4 dpf (Protas and Leontieva 1992). In *X. laevis*, adrenergic tonus develops at 3 dpf, peaking at around 4–5 dpf (Jacobsson and Fritsche 1999), which corresponds to the onset of tachycardia in response to acute hypoxic in the present study. In larval *X. laevis*, catecholamines and enzymes responsible for catecholamine synthesis have been detected in the heart, kidneys, and the rest of the body immediately after hatching (Kloberg and Fritsche 2002). Collectively, these findings suggest that catecholamines produced both within and outside the heart are potential sources for early f_H regulation before sympathetic innervation develops.

f_H responses to acute hypoxia have been documented extensively in vertebrates. However, in developing anurans, what exactly comprises the f_H response to acute hypoxia remains controversial. In the present study we found strong and consistent tachycardia in response to acute hypoxia in larvae between 5 and 25 dpf (Fig. 1). Feder and Wassersug (1984) also reported increased f_H in response to declining aquatic PO_2 in free-swimming *X. laevis* with access to air, although the effect of acute hypoxia was rather modest. In contrast, bradycardia as an acute hypoxic response has also been found using the same species (Orlando and Pinder 1995; Fritsche and Burggren 1996) although in both of these studies, anesthesia and more invasive methods were involved. In the present study, unanaesthetized larvae were lightly restrained and had no access to air during the very brief measurement period, but had enough space for normal buccal pumping behaviors. In addition to measurement protocol, length of hypoxic exposure could also cause variation. In the neotenous axolotl, NaCN, a chemical that mimics hypoxia, decreases f_H initially, but then induces tachycardia later on during acute hypoxic incubation (McKenzie and Taylor 1996).

Table 1 Regression relationships of heart rate (f_H), stroke volume (SV) and cardiac output (CO) against body mass at different PO_2 for chronic and acute exposures in developing *Xenopus laevis*

Variable	Chronic PO_2 (mmHg)	Acute PO_2 (mmHg)	Equation	n	r^2	p
f_H	150	150	$2.19 \cdot mass^{-0.04}$	78	0.20	<0.001
		110	$2.26 \cdot mass^{-0.04}$	78	0.21	<0.001
		70	$2.27 \cdot mass^{-0.04}$	78	0.23	<0.001
	110	150	$2.16 \cdot mass^{-0.01}$	77	0.02	0.192
		110	$2.22 \cdot mass^{-0.01}$	77	0.01	0.333
		70	$2.25 \cdot mass^{-0.02}$	77	0.11	0.003
SV	150	150	$0.06 \cdot mass^{1.23}$	78	0.89	<0.001
		110	$0.33 \cdot mass^{1.09}$	78	0.91	<0.001
		70	$0.41 \cdot mass^{1.06}$	78	0.89	<0.001
	110	150	$-0.04 \cdot mass^{1.23}$	77	0.89	<0.001
		110	$0.14 \cdot mass^{1.19}$	77	0.92	<0.001
		70	$0.28 \cdot mass^{1.10}$	77	0.94	<0.001
CO	150	150	$-0.75 \cdot mass^{1.19}$	78	0.88	<0.001
		110	$-0.41 \cdot mass^{1.05}$	78	0.90	<0.001
		70	$-0.33 \cdot mass^{1.02}$	78	0.89	<0.001
	110	150	$-0.89 \cdot mass^{1.22}$	77	0.88	<0.001
		110	$-0.64 \cdot mass^{1.18}$	77	0.91	<0.001
		70	$-0.47 \cdot mass^{1.08}$	77	0.92	<0.001

Developmental studies of f_H with a longer recording time and a comparison of different methods may be necessary to interpret these conflicting data. Finally, epigenetic, trans-generational effects of hypoxic exposure on larval vertebrate responses to hypoxia are emerging (e.g. Ho 2008; Ho and Burggren 2010), and will need to be accounted for in the future.

One of the potential benefits of tachycardia during aquatic hypoxia for air breathers may be an increase in perfusion of the air-breathing organ, which secures higher O_2 supply to the heart and other tissues. Indeed, in most air-breathing fish, especially obligate air breathers, and amphibians, bradycardia (a common cardiac response to acute hypoxia in water-breathing animals, Farrell 2007), generally disappears and is replaced by either tachycardia or a lack of chronotropic response (McKenzie and Taylor 1996; Sanchez et al. 2001; Andersen et al. 2003; Perry et al. 2005; Farrell 2007). The hypoxic tachycardia and increased cardiac output observed here also coincide with the onset of increased air-breathing frequency in response to acute hypoxia (Pan and Burggren 2010). However, in amphibians, changes in the perfusion of air-breathing organs can also be achieved by intracardiac shunting and alteration in vascular resistance. Hence, the emergence of air breathing cannot adequately explain the disappearance of hypoxic bradycardia during evolution or during development. In addition to tachycardia during acute hypoxia in the current study, larvae at early stages also showed increased SV, resulting from an increased EDV (Fig. 3). Increased EDV, suggesting a greater venous return, with maintained ESV collectively indicates that hearts of early larvae exhibit aspects of a Frank–Starling relationship, an observation also made on this species by Warburton and Fritsche (2000). However, the present results show for the first time that this relationship may influence acute cardiac responses to hypoxia.

Effects of chronic hypoxic acclimation

In the present study, rearing larvae in chronic moderate hypoxia (110 mmHg) had no effect on f_H when plotted against age. However, SV and CO were attenuated when measured at 7 and 10 dpf (Fig. 4). Interestingly, SV and CO responses to acute hypoxia were also found in the same ages (Fig. 3), suggesting a window of cardiac development more susceptible to hypoxia—whether acute or chronic. No previous study has examined the effect of chronic hypoxic acclimation on cardiac performance during acute hypoxic exposure in larval amphibians, but similar reductions in cardiac function have been documented in the embryonic chicken (Sharma et al. 2006) and in the adult Atlantic cod, *Gadus morhua* (Petersen and Gamperl 2010a). However, neither metabolic rate nor growth is affected by chronic hypoxia in early larval *X. laevis* (Territo and Altimiras 1998; Pan and Burggren 2010), a decrease in CO probably has little or no effect on tissue oxygenation. In fact, the very small embryos and larvae of vertebrates achieve a significant proportion of their gas exchange through direct

diffusion (see Burggren 2005) reducing the impacts of decreased cardiac output.

With respect to mechanism for heart performance modulation, whether acute or chronic hypoxia in the current study exerts direct effects on the heart tissue or indirectly affects heart performance through neural or hormonal modulation is still unknown. Studies using larval *X. laevis* (Fritsche and Burggren 1996) and adult *G. morhua* (Petersen and Gamperl 2010b) both suggest a direct effect of hypoxia on the heart.

Cardiac scaling with body mass

Cardiac scaling patterns in early larval *X. laevis* (*b* of −0.04, 1.23 and 1.19 for f_H, SV and CO, respectively) align favorably with previously reported values in the same species (Hou and Burggren 1995a, b; Orlando and Pinder 1995). However, these scaling coefficients contrast with the so-called "universal" values predicted (*b* of −0.25, 1 and 0.75 for f_H, SV and CO) based on the data collected in mammals (West et al. 1997). This suggests that during early development in *X. laevis*, f_H is relatively maintained across the range of body mass investigated, and that the SV and CO increase faster than tissue growth. As mentioned, direct diffusion is thought to be sufficient to supply developing amphibian larvae at early stages with all their O_2 need (Territo and Altimiras 1998, 2001; Burggren 2005). If cardiac function is not critical to maintain O_2 consumption during development, the commonly observed rapid increase in cardiac output relative to the increasing body mass may be essential for the transport of nutrients, signaling molecules and materials to maintain larval homeostasis, or participating in angiogenesis (Burggren 2005; Branum et al. submitted).

In addition to a different cardiac scaling slope in *X. laevis*, the relationship between f_H (measured at a PO_2 of 150 and 110 mmHg) and mass evident in normoxia was missing in chronic hypoxia (Table 1); that is, the developmental pattern of f_H is independent of changes in mass in hypoxic larvae. The differences in f_H scaling pattern between normoxic- and hypoxic-reared larvae mainly occur later in larval development, when hypoxic larvae show higher f_H (Fig. 5a). This may result from the cumulative effect of hypoxia on f_H. In addition, the difference in f_H scaling pattern may also be important for the CO compensation in hypoxic larvae, because a very similar CO scaling coefficient is found in our study (*b* of 1.19 and 1.18 for normoxic and hypoxic larvae measured at their rearing PO_2). The effects of O_2 environment on cardiac scaling relationship with mass have not been investigated before. However, pH and temperature have shown to modulate metabolic scaling with mass in adult fishes (Ohlberger et al. 2007; Killen et al. 2010; Vaca and White 2010). Our results indicate that not only metabolic, but also cardiac allometry can be shaped by environment, and that the *b* of either 0.75 for oxygen consumption (Territo and Altimiras 1998), or −0.25 for f_H, is not evident in larval amphibians.

Conclusions

The developmental pattern of f_H in newly hatched *X. laevis* shows an early, sharp increase after the onset of heartbeat to 4 dpf, which is then followed by a gradual decrease. CO and SV both increase dramatically during development with body mass. When exposed to acute hypoxia, larvae mainly increase f_H, which in turn causes an increase in mass-specific CO. Development of cholinergic and adrenergic receptors on the heart and cardiac and non-cardiac catecholamines are possible candidates controlling f_H in response to acute hypoxia during early larval life. Rearing in chronic hypoxia decreases SV and CO during early development (7 and 10 dpf) and modifies f_H allometry.

Our study additionally shows that within the first week of development, not only the cardiac system itself, but also the regulatory system controlling cardiac functions have formed. Both the developmental trajectory of this early cardiac control system and cardiac scaling relationship with *M* are developmentally plastic, and can be modified by long-term alteration of ambient O_2. Further comparative and mechanistic studies of ontogenetic changes of the regulatory system may provide insight into evolution and importance of early cardiac control and the constraints of physiological allometry.

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