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RESEARCH ARTICLE

The ontogeny of regulatory control of the rainbow trout (*Oncorhynchus mykiss*) heart and how this is influenced by chronic hypoxia exposure

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SUMMARY

Salmonid embryos develop in cool waters over relatively long periods of time. Interestingly, hypoxic conditions have been found to be relatively common in some nesting sites (redds). The goals of this study were to determine the ontogeny of cardiac regulation in rainbow trout early life stages and how this is influenced by chronic hypoxia. The heart rate response to cholinergic and adrenergic receptor stimulation or inhibition was measured in individuals reared in normoxic (100% O_2 saturation) or hypoxic (30% O_2 saturation) conditions from fertilization to embryonic stages 22, 26 and 29, and larval stages 30 and 32. In normoxia, heart rate increased in response to β-adrenergic receptor stimulation (isoproterenol) as early as embryonic stage 22, and decreased with the antagonist propranolol after this stage. Cholinergic stimulation (acetylcholine) was ineffective at all stages, but atropine (acetylcholine antagonist) increased heart rate at larval stage 32. This demonstrates that cardiac β-adrenergic receptors are functional at early life stages, while cholinergic receptors are not responsive until after hatching. Collectively, embryos had cardio-acceleration control mechanisms in place just after the heartbeat stage, while cardio-inhibitory control was not functional until after hatching. Chronic hypoxia exposure triggered bradycardia, increased the response to adrenergic stimulation in embryos and larvae, and delayed the onset of cholinergic control in larvae. In non-motile stages, therefore, survival in chronic low oxygen may depend on the ability to alter the cardiac ontogenic program to meet the physiological requirements of the developing fish.

Key words: adrenergic stimulation, cholinergic stimulation, heart rate, hypoxia, ontogony of cardiac regulation, trout embryos.

INTRODUCTION

The regulation of cardiac function in adult fish occurs through intrinsic, neural or humoral mechanisms (for reviews, see Satchell, 1991; Farrell and Jones, 1992). As in other vertebrates, heart rate is primarily controlled by variable inputs of adrenergic and cholinergic stimulation, corresponding to the sympathetic and parasympathetic branches of the autonomic nervous system, respectively (Farrell and Jones, 1992). There is limited information on how this control develops during early development as most studies have focused on post-hatching larvae. These previous studies demonstrate that adrenergic and cholinergic receptors respond to stimulation by producing chronotropic effects but these responses are not fully matured (Holeton, 1971; McDonald and McMahon, 1977; Burggren and Pinder, 1991; Orlando and Pinder, 1995; Fritsche and Burggren, 1996; Fritsche, 1997; Crossley et al., 2003b; Lindren and Altimiras, 2009). In the golden mullet (*Liza auratus*), for example, adrenergic but not cholinergic receptors were reported to be functional 6 days after hatching (Balashov et al., 1991), while the zebrafish (*Danio rerio*) heart responded to adrenergic and cholinergic stimulation 3 and 4 days after hatching, respectively (Schwerte et al., 2006). Thus, at the larval stage adrenergic receptors are functional in some fish species, but how early in the development of the heart and circulatory system do these regulatory pathways first appear? Further, how plastic is embryonic cardiac development in response to environmental perturbation?

One condition that may significantly impact the ontogeny of cardiac regulatory control mechanisms in fish is environmental hypoxia. Dissolved O_2 (DO) levels have been found to be highly variable in salmonid nesting sites (redds), with levels as low as 19% reported (Coble, 1961; Peterson and Quinn, 1996; Youngson et al., 2004). In addition, the encapsulated, non-motile embryos may remain under these conditions for up to 5 months prior to emergence (Youngson et al., 2004). Previous work has demonstrated that embryonic development of teleost fish is extremely sensitive to environmental conditions (Ørnsrud et al., 2004) and that chronic hypoxia exposure delays development in fish (Shumway et al., 1964; Hamor and Garside, 1976; Ciuhandu et al., 2005; Spicer and Burggren, 2003; Bagatto, 2005; Miller et al., 2008). The reduction in developmental rate represents an adaptive response to reduce O_2 demand (Miller et al., 2008). This means, then, that there is a significant period of time for embryonic development in these temperate fishes to be influenced by environmental factors. This is quite different from the development of the hypoxia-tolerant, tropical zebrafish, where development occurs very rapidly and the larvae are free swimming within 3 days (Kimmel et al., 1995). Therefore, although zebrafish are used as a model species for understanding the ontogeny of various physiological systems in fish and more generally in vertebrates, they may not be representative of the thousands of fish species that develop slowly in cold temperate waters.

It is well documented that adult salmonids respond to acute hypoxia with bradycardia, but maintain cardiac output by a compensatory increase in stroke volume (Holeton and Randall, 1967; Randall, 1982). However, in larval salmonids (i.e. after hatching but before feeding) this response is absent, and it has been reported that acute hypoxia results in an increase in heart rate or tachycardia

(Holeton, 1971; McDonald and McMahon, 1977). Accordingly, Holeton (Holeton, 1971) speculated that the vagal reflex-mediated bradycardia is not yet functional in rainbow trout up to 8 days after hatching. Although 40 years have passed since Holeton's classic 1971 study, there have been no follow-up investigations of the hypoxic cardiac response in either embryonic or larval rainbow trout. The hypoxic bradycardia in hatched, 4 days post-fertilization (d.p.f.) zebrafish is blocked in muscarinic cholinergic receptor knockdowns (Steele et al., 2009), indicating that the heart is under inhibitory vagal control by this stage of development. Similarly, heart rate variability of emu (*Dromiceius novaehollandiae*) hatchlings in hypoxia was abolished after cholinergic receptor blockade with atropine (Shah et al., 2010). While in early larval African clawed frog (*Xenopus laevis*), hypoxia has a direct inhibitory effect on heart rate, in later stages, active regulation of stroke volume and cardiac output occurs when the neural and humoral cardiovascular controls have matured (Fritsche, 1997). Together, these results demonstrate that the response of the vertebrate heart to hypoxia exposure is dependent on developmental stage.

It is unknown when cardiac responsiveness to adrenergic or cholinergic agents first develops, or the importance of these factors in regulating cardiac function during environmental hypoxia in the early life of a salmonid. Therefore, the ontogeny of cardiac regulation and the influence of chronic hypoxia were examined in the rainbow trout. It was hypothesized that heart rate in embryos and larvae is controlled by the development of adrenergic and cholinergic input, and its interaction with environmental O_2. If this is the case, then cardiac adrenergic and cholinergic receptor stimulation or inhibition should produce chronotropic changes that depend on development, and chronic hypoxia exposure should alter the responsiveness of receptors. To test this, the heart rate response to cholinergic and adrenergic receptor stimulation or inhibition was measured in trout embryos and larvae reared in normoxia or hypoxia (30% O_2 saturation) from fertilization to stages 22, 26 and 29 (before hatching), and 30 and 32 (after hatching).

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) embryos were obtained from Rainbow Springs Trout Farm in Thamesford, ON, Canada, on the day of fertilization and transported to the Hagen Aqualab, University of Guelph. Embryos were held in meshbottom Heath trays (62.9×52.1×6 cm l×w×d) throughout the incubation period and shielded from light, with a continuous flow (4 ml min^{-1}) of local untreated well water [10°C, 10 mg O_2 l^{-1}, pH 7.9, water hardness 411 mg $CaCO_3$ l^{-1}, ion concentrations (mmol l^{-1}): 2.6 Ca^{2+}, 1.5 Cl^-, 1.5 Mg^{2+}, 0.06 K^+ and 1.1 Na^+]. Separate groups of embryos were used for each series of experiments. Each group was derived from a spawning event between three females and three males. Hatching occurred 29–31 d.p.f. for all groups. Developmental stages were designated according to Vernier (Vernier, 1969).

Experimental protocol

The heart rate of embryos and larvae was determined *in vivo* by direct observation under a dissecting microscope and involved counting the number of contractions for 60 s. Measurements were taken in glass-bottomed Petri dishes (MatTek Co., Ashland, MA, USA) where the water temperature (10±0.3°C) was maintained through a cooling stage connected to a bath circulator (Fisher Isotemp 3016, Pittsburgh, PA, USA). Measurements were taken at chronological times 15, 22, 29, 36 and 43 d.p.f., corresponding to Vernier (Vernier, 1969) developmental stages 22, 26, 29, 30 and 32.

Embryos and larvae were exposed to drugs *via* a water bath. Preliminary experiments showed that 30 min was more than sufficient time for heart rate to stabilize after drug exposure. All drugs were obtained from Sigma-Aldrich (St Louis, MO, USA). For post-hatching larvae experiments (stages 30 and 32), tricaine (MS-222; 20 mg l^{-1}) was added to the exposure water to reduce body movement. MS-222 is a neuromuscular blocker, and has been used to analyze the cardiac activity of amphibian and fish larvae (Pelster and Bemis, 1991; Pelster and Burggren, 1991; Fritsche and Burggren, 1996; Mirkovic and Rombough, 1998), and of adrenergic and cholinergic cardiac responsiveness of fish larvae (Hsieh and Liao, 2002; Bagatto, 2005; Schwerte et al., 2006; Steele et al., 2009). In a previous study, this concentration of MS-222 was found to have no significant effect on heart rate in rainbow trout larvae (Mirkovic and Rombough, 1998).

Series I: adrenergic and cholinergic cardiac response

To pharmacologically characterize cardiac cholinergic and adrenergic receptors, we used a concentration of agonist previously found to produce chronotropic effects in zebrafish embryos and larvae (Schwerte et al., 2006). Embryos or larvae reared in normoxia were placed individually in water-filled Petri dishes. After 30 min, control values of heart rate were measured. Each larva or embryo was then placed in a solution of either 0.1 mmol l^{-1} isoproterenol (β-adrenergic receptor agonist) or 0.1 mmol l^{-1} acetylcholine (cholinergic receptor agonist). Following 30 min of drug incubation, heart rate was measured. To confirm agonist specificity, each individual was then placed in a fresh solution of 0.1 mmol l^{-1} isoproterenol combined with 1 mmol l^{-1} propranolol (β-adrenergic receptor antagonist) or 0.1 mmol l^{-1} acetylcholine combined with 1 mmol l^{-1} atropine (cholinergic receptor antagonist). After 30 min, heart rate was measured. Acetylcholine is a general cholinergic receptor agonist (nicotinic and muscarinic receptors) readily degraded by acetylcholinesterase. Therefore, to ensure that a negative response to acetylcholine was not due to degradation of the drug, the same protocol was repeated with 0.1 mmol l^{-1} muscarine (stable muscarinic receptor agonist) in place of acetylcholine. A series of trial experiments were also conducted to determine the responsiveness of cholinergic and adrenergic receptors to low drug concentrations (0.001 mmol l^{-1} agonist ±0.01 μmol l^{-1} antagonist). Finally, to ensure that following drug exposure embryos or larvae fully recovered, we followed heart rate for up to 2 h after the drug was removed.

To determine the receptor (adrenergic and cholinergic) tone on heart rate in chronic normoxia, embryos and larvae were serially incubated in autonomic receptor antagonists as described previously (Altimiras et al., 1997; Crossley et al., 2003a). Baseline heart rate was determined following a 30 min control period. The embryo or larva was then placed in a solution of atropine (0.1 mmol l^{-1}) for 30 min to remove the cholinergic receptor agonist. Complete blockade of receptors was then achieved by placing the individual in a fresh solution of atropine and propranolol (0.1 mmol l^{-1}) combined. Heart rate was measured after 30 min. This order of drug exposure was used as Altimiras and colleagues (Altimiras et al., 1997) suggest that if propranolol is used as a β-receptor antagonist it should be applied after atropine because it enhances cholinergic tone in adults. Adrenergic and cholinergic tone were calculated using formulas modified from Altimiras et al. (Altimiras et al., 1997) so that an absolute change in heart rate (Δf_H) is calculated instead of percentage tone. Percentage tone is not comparable to that in adult

fish because tone is still developing in the embryos while it is already established in adult fish. The formulas used were:

$$\text{Adrenergic tone } (\Delta f_H) = f_{H,\text{intrinsic}} - f_{H,\text{atropine}}, \quad (1)$$

$$\text{Cholinergic tone } (\Delta f_H) = f_{H,\text{atropine}} - f_{H,\text{control}}, \quad (2)$$

where $f_{H,control}$ is the control (baseline) heart rate, $f_{H,atropine}$ is the heart rate after muscarinic receptor blockade and $f_{H,intrinsic}$ is the heart rate after muscarinic and β-adrenergic receptor blockade.

Series II: adrenergic and cholinergic cardiac response and chronic hypoxia

To determine whether the responsiveness of cardiac cholinergic and adrenergic receptors is altered with chronic hypoxia, embryos were reared in normoxia (100% O_2 saturation) or chronic hypoxia (30% O_2 saturation) from the day of fertilization. This level of hypoxia has been recorded over extended periods of time in salmonid redds (Peterson and Quinn, 1996). To control water O_2 levels, N_2 gas was bubbled into a header tank (~50 l) connected to the Heath tray containing the embryos. DO levels in the water were monitored using a Luminescent Dissolved Oxygen (LDO^{TM}) meter (model HQ40d-LDO101, Hach Co., Loveland, CO, USA). Hypoxic exposure results in developmental delay (Hamor and Garside, 1976) and therefore measurements for this group were stage-matched with the normoxic group. Fig. 1 is a picture of two larvae from the same fertilization event but reared under chronic hypoxia or control conditions.

In the experimental chamber, hypoxic water was maintained by mixing air and N_2 gas using a gas-mixing pump (Wösthoff pump, Calibrated Instruments Inc., Ardsley, NY, USA). Similarly, normoxic water was maintained by air input to the gas-mixing pump. DO levels in the experimental chamber were held constant by placing an air-tight lid over the chamber. A mixture of nitrogen and air from the gas-mixing pump was introduced into the chamber *via* small plastic tubing, and flowed out *via* an outlet hole. To determine autonomic tone on heart rate regulation in normoxia- and hypoxia-reared embryos and larvae, the same protocol was repeated as in Series I, except that the concentration of antagonists was 1 mmol l^{-1} to ensure complete receptor blockade.

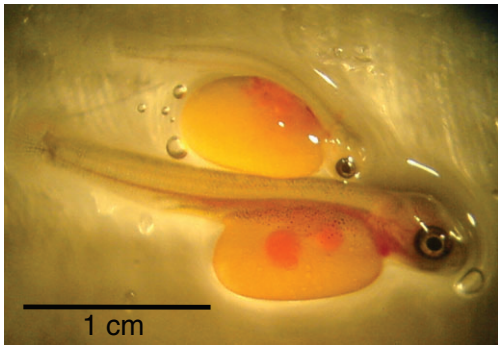

Fig. 1. Chronic hypoxia (30% O_2 saturation) incubation from fertilization results in delayed development and growth in trout embryos. Photo was taken 41 days post-fertilization (d.p.f.), with the normoxia-reared (100% O_2 saturation) larva at developmental stage 32 (bottom) and the hypoxia-reared larva at developmental stage 30 (top). These embryos are the result of the same fertilization event.

Statistical analyses

Statistical analyses were performed using Minitab 15.1 (Minitab Inc., State College, PA, USA). One-way repeated measures ANOVA with Tukey *post hoc* analyses were used to determine statistically significant differences in heart rate between treatments within a given developmental stage for characterization of receptors in Series I. Differences in receptor tone (cholinergic or adrenergic) between developmental stages in Series I were compared using one-way ANOVA with Tukey *post hoc* analyses. The normoxia and hypoxia baseline heart rates from Series II were compared within and between developmental stages using a two-way ANOVA (O_2 treatment, developmental stage) with a Tukey *post hoc* analysis. For Series II data, two-way ANOVA (O_2 treatment, developmental stage) with Tukey *post hoc* analyses were used to determine differences in receptor tone (cholinergic and adrenergic) between normoxia and hypoxia at a given developmental stage, and between developmental stages within normoxia or hypoxia. The significance level for all statistical tests was set at $P<0.05$. All values are presented as means ± s.e.m.

RESULTS

Experimental approach

No mortalities occurred as a result of drug exposure. Following drug exposure, heart rate in embryos recovered, although the recovery time was stage dependent. At stage 22, recovery from propranolol occurred by 30 min (normal recovery period), but after stage 22, heart rate gradually returned to control level within 2 h. For comparison, the concentrations of drugs were chosen to match those in a previous study where zebrafish embryos and larvae were bathed in solutions containing either 0.1 mmol l^{-1} agonist or 0.1 mmol l^{-1} agonist plus 1 mmol l^{-1} antagonist (Schwerte et al., 2006). The chronotropic responses observed in response to various drugs were repeatable over a range of concentrations from 0.001 (N=3–5, data not shown) to 0.1 mmol l^{-1} (see below).

Series I: adrenergic and cholinergic cardiac response

Embryos exposed to the adrenergic receptor agonist isoproterenol showed a significant increase in heart rate at stage 22 ($P<0.0001$) (Fig. 2). This response was not observed in embryos at stages 26 and 29, but was apparent after hatching at stage 30 (P=0.012) and stage 32 (P=0.047). Co-incubation with the adrenergic receptor antagonist propranolol lowered heart rate and blocked the positive chronotropy induced by isoproterenol at all stages ($P<0.001$). The influence of propranolol increased with development. Propranolol exposure induced a significant decrease in heart rate of 8%, 17%, 16%, 60% and 68% at stages 22, 26, 29, 30 and 32, respectively (Fig. 2). Acetylcholine had no significant effect at any stage (Fig. 3). Exposure to atropine induced a significant increase in heart rate relative to acetylcholine alone at stage 30 (P=0.0085), and at stage 32 the increase in heart rate above control was also significant (P=0.0012). Similarly, muscarine had no significant effect on heart rate at any stage (N=3, data not shown).

At all stages tested, heart rate significantly decreased following adrenergic receptor blockade (Fig. 4). The bradycardia was weak at stage 22 (change of –6 beats min^{-1}), strong at stage 26 (–11 beats min^{-1}) and strongest at stages 30 and 32 (–16 and –17 beats min^{-1}, respectively). From stage 22 to 30, cholinergic receptor blockade had no significant effect on heart rate. At stage 32, however, a significant tachycardia (+7 beats min^{-1}) occurred in response to cholinergic receptor inhibition ($P<0.01$).

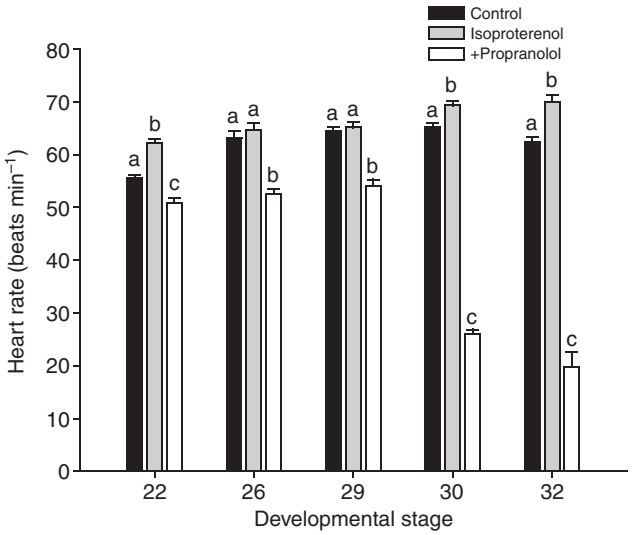

Fig. 2. The trout heart is responsive to adrenergic stimulation during early embryonic development. Heart rate of normoxia-reared (100% O_2 saturation) rainbow trout embryos and larvae was measured in control conditions or following exposure to 0.1 mmol l^{-1} isoproterenol or combined 0.1 mmol l^{-1} isoproterenol + 1 mmol l^{-1} propranolol at different stages of development (Series I). Data are presented as means + s.e.m. (N=6). Different letters indicate statistically significant differences between treatments at a given stage (P<0.05).

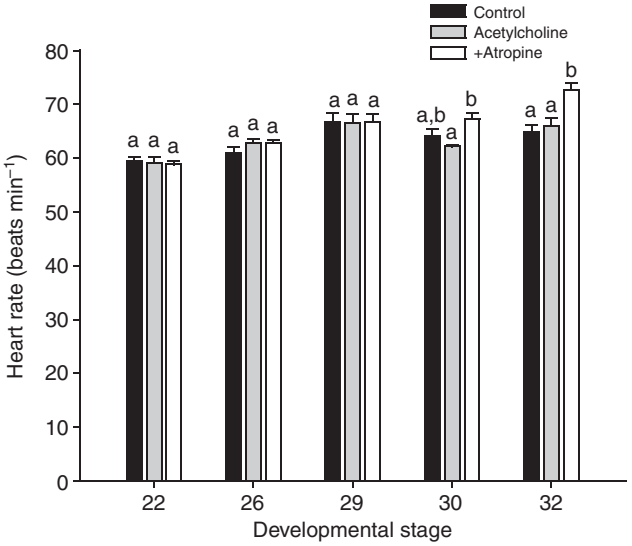

Fig. 3. The trout heart is controlled by cholinergic input after hatching. Heart rate of normoxia-reared (100% O_2 saturation) rainbow trout embryos and larvae was measured in control conditions or following exposure to 0.1 mmol l^{-1} acetylcholine or combined 0.1 mmol l^{-1} acetylcholine and 1 mmol l^{-1} atropine at different stages of development (Series I). Data are presented as means + s.e.m. (N=6). Different letters indicate statistically significant differences between treatments at a given stage (P<0.05).

Series II: adrenergic and cholinergic cardiac response with chronic hypoxia

DO levels in the chronic hypoxia treatment were relatively constant over the duration of the experiment (44 days, 30±2% saturation, data not shown). There was a significant interactive effect between development and O_2 exposure on adrenergic (P<0.001) and cholinergic (P=0.003) tone. Developmental stage (P<0.001) and O_2 treatment (P<0.001) had significant effects on adrenergic tone (Fig. 5A). In normoxia-reared individuals, propranolol elicited a similar developmental response to that described above (compare Fig. 4 and Fig. 5A), where the magnitude of the inhibition of heart rate was greater at stages 30 (change of –31 beats min^{-1}) and 32 (–38 beats min^{-1}), relative to that at earlier stages (P<0.001). It should be noted that heart rate was not significantly different from baseline in the presence of propranolol at stage 22 in Fig. 5A, but was significantly different in Fig. 4. In hypoxia-reared individuals, the decrease in heart rate in response to propranolol followed a similar pattern, increasing in magnitude from stage 22 (–11 beats min^{-1}, P<0.001) to stage 32 (–57 beats min^{-1}, P<0.001). The change in heart rate induced by propranolol was significantly greater (between 1.5- and 2.6-fold) in hypoxia relative to normoxia at stages 26 (P=0.002), 29 (P<0.001), 30 (P=0.027) and 32 (P<0.001).

Developmental stage (P<0.001), but not O_2 treatment (P=0.159), had a significant effect on cholinergic tone (Fig. 5B). Individuals reared in normoxia did not consistently respond to cholinergic blockade until stage 32 (note similar results presented in Fig. 3), where atropine elicited a significant increase in heart rate in normoxia (P<0.001, change of +10 beats min^{-1}) but not hypoxia. Atropine treatment did not change heart rate of hypoxic individuals at any stage.

Developmental stage (P<0.001) and O_2 treatment (P<0.001) had significant effects on heart rate. There was a significant interactive effect between development and O_2 exposure on heart rate (P<0.001). In normoxic embryos, heart rate significantly increased by 26% from stage 22 to 26 (P<0.001) (Fig. 6). After this initial rise, heart rate remained relatively constant from stage 26 to 29. At stage 30 there was a small but significant drop in heart rate that was maintained at stage 32 (P=0.029). In hypoxic embryos, heart rate remained low and constant between stages 22 and 29, with heart rate at stages 26 and 29 being significantly lower (19–20%) relative to that of normoxic embryos (P<0.001). After stage 29, heart rate significantly increased (11%) (P<0.001) to match the rate of normoxic larvae at stage 30, reaching a maximum at stage 32.

DISCUSSION

This is the first investigation of the responsiveness of cardiac adrenergic and cholinergic receptors throughout early development in a salmonid. The results of this study indicate that autonomic control systems of the trout heart are operational at early embryonic life stages. In addition, there is evidence that chronic hypoxia has profound effects on the developmental trajectory of cardiac regulatory mechanisms. The results provide support for stage dependency in chronotropic responses to adrenergic and cholinergic receptor stimulation or inhibition. Adrenergic receptors were functional at all stages; however, cholinergic receptors were functional only after hatching (Fig. 7). Chronic hypoxia increased adrenergic receptor control of heart rate, and delayed the onset of cholinergic control. These two mechanisms may be the key hypoxia acclimation strategies that non-motile, hatched larvae use to regulate cardiac function and circulation when faced with a low oxygen environment and no means of escape. The results support the hypothesis that heart rate in embryos and larvae is controlled by the development of adrenergic and cholinergic input, and its interaction with environmental O_2. Moreover, these results validate the speculation by Holeton (Holeton, 1971) that the drop in resting

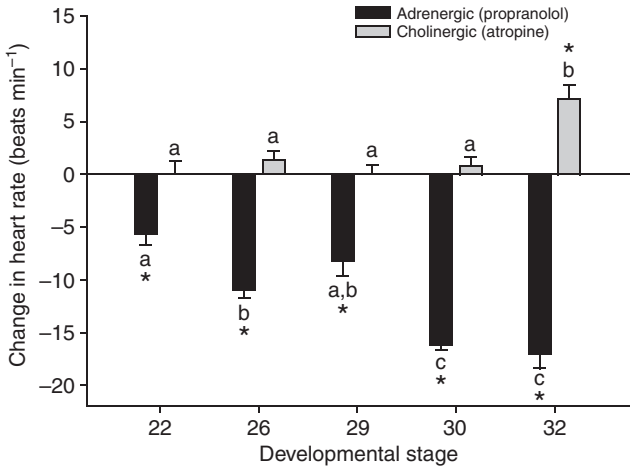

Fig. 4. Tonic adrenergic regulation of the trout heart is significant in embryos and larvae, while tonic cholinergic regulation of heart rate becomes apparent only after hatching. The absolute change in heart rate was obtained by measuring heart rate under control conditions and then transferring the embryo or larva into a solution of atropine (0.1 mmol l^{-1}) for 30 min to remove the cholinergic receptor agonist. Complete blockade of receptors was then achieved by placing the individual in a fresh solution of atropine and propranolol (0.1 mmol l^{-1}) combined. Data are presented as means + s.e.m. (N=6). Different letters indicate statistically significant differences between developmental stages for adrenergic or cholinergic tone and asterisks represent significant drug response from baseline (P<0.05).

heart rate after hatching is due to the onset of inhibitory nervous control of the heart *via* the vagus nerve.

The development of cardiac regulatory control

Our findings show that the trout heart is capable of adrenergic regulation shortly after the start of cardiac contractions (Fig. 7). A positive chronotropic effect from β-adrenergic receptor stimulation was observed as early as stage 22 and this response was abolished by the bradycardic response to propranolol. At later stages, β-adrenergic receptor inhibition had a greater influence on heart rate, indicating an increased adrenergic influence on cardiac function. The adrenergic receptor stimulation in this study is similar to the positive chronotropic effect of adrenaline in adult rainbow trout (Farrell et al., 1986). Schwerte and colleagues (Schwerte et al., 2006) found a positive chronotropic response to adrenergic receptor stimulation in early stages of *D. rerio*, but only after hatching.

The positive chronotropic effect induced by isoproterenol at stages 22, 30 and 32 suggests a functional β-adrenergic receptor population that is not saturated, indicating a greater scope for tachycardia. At stages 26 and 29 the ineffectiveness of isoproterenol suggests that the majority of β-adrenergic receptor sites are likely saturated and this could be due to ongoing developmental processes including a change in the number of β-adrenergic receptors, receptor affinity or the signaling pathways downstream from the receptors. An increase in β-adrenergic receptor density and cAMP production occurs with development in fetal mice (Chen et al., 1982). Thus, developmental changes in the coupling of β-adrenergic receptor activation to messenger systems downstream can also be responsible for altered chronotropic responses to drugs across development. Similarly, in chicken embryos, the decrease in isoproterenol sensitivity of the ventricle between days 16 and 21 of embryonic development is in agreement with the loss of β-adrenergic receptors from day 15 to 19, and indicates receptor internalization or

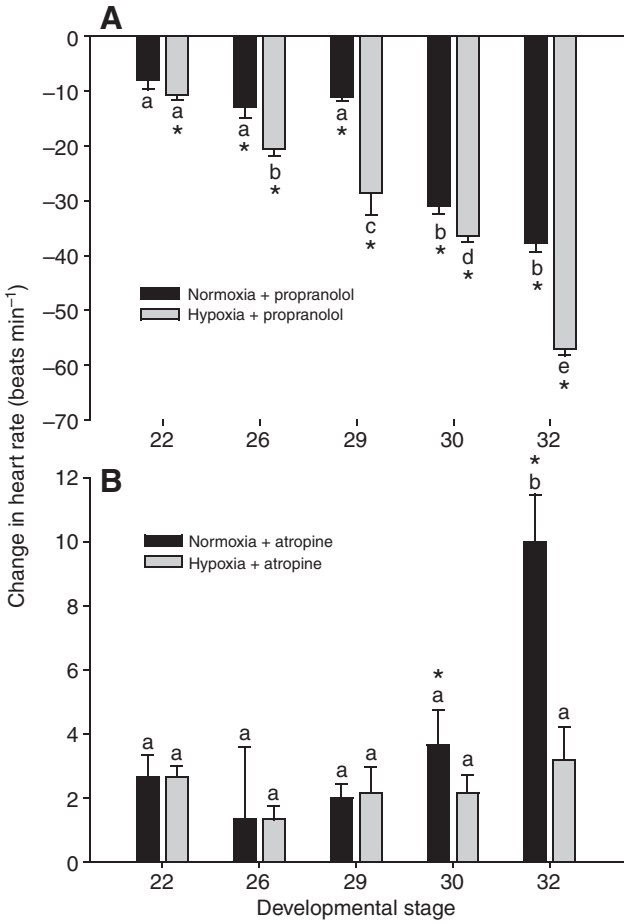

Fig. 5. Hypoxia exposure increases the adrenergic tone of the embryonic trout heart throughout development. Cardiac autonomic tone of normoxia- (100% O_2 saturation) and hypoxia-reared (30% O_2 saturation) rainbow trout embryos and larvae was measured as changes in heart rate in response to (A) adrenergic receptor blockade by 1 mmol l^{-1} propranolol (adrenergic tone) and (B) cholinergic receptor blockade by 1 mmol l^{-1} atropine (cholinergic tone) at different stages of development (Series II). Data are presented as means + s.e.m. (N=6). Different letters indicate statistically significant differences between treatments at a given stage, and between stages within normoxia or hypoxia. Asterisks represent significant drug response from control normoxic or hypoxic values (P<0.05).

downregulation (Higgins and Pappano, 1981; Lindgren and Altimiras, 2009).

The maturation of an adrenergic tone on heart rate regulation was inferred from the intensified bradycardia response to propranolol after hatching. Indeed, higher adrenaline levels after hatching relative to previous stages were found in the head and heart tissues of rainbow trout larvae (Meyer and Sauerbier, 1977). In addition, nervous control of catecholamine secretion from chromaffin cells of trout was reported as early as pre-hatching stage 28 (Gallo and Civinini, 2005). Therefore, the higher adrenergic tone on heart rate can presumably be related to increasing levels of circulating catecholamines. The increase in adrenergic tone observed over developmental time is likely a result of an increase in body mass, metabolic rate and critical oxygen levels that impose an additional demand on the convectional transport of oxygen (Rombough, 1988b). Younger embryos may be less dependent on the circulatory system for oxygen, as their needs may be met by diffusion (Rombough, 1988a). In rainbow trout larvae, the contribution of the

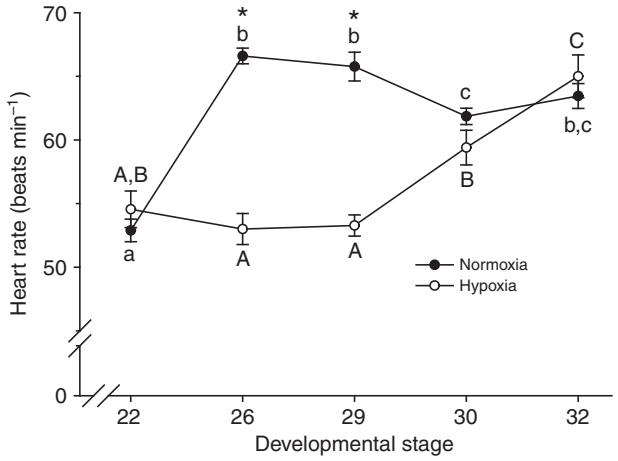

Fig. 6. Developmental pattern of heart rate in rainbow trout embryos and larvae reared in normoxia (100% O_2 saturation) or hypoxia (30% O_2 saturation) (Series II). Data are presented as means ± s.e.m. (N=9–22). Different letters (lowercase, normoxia; uppercase, hypoxia) indicate statistically significant differences in heart rate between developmental stages and asterisks represent significant differences between normoxia and hypoxia (P<0.05).

gills (convection) relative to the skin (diffusion) to oxygen uptake progressively increases to 50% of total oxygen uptake rate by 23±2 days post-hatching in soft water and 28±3 days post-hatching in hard water (Fu et al., 2010). This was defined as the time at which oxygen uptake transitions from the skin to the gills (Fu et al., 2010). The timing of these respiratory changes parallels the results of the present study, where the regulatory pathways of cardiac function were fully mature 14 days after hatching.

Although functional β-adrenergic receptors are present, it does not imply that stimulation can occur from the sympathetic nervous system. The participation of sympathetic nerves in the regulation of heart rate in early developmental stages of fish is unknown. Embryos of the chicken (*Gallus gallus*) and common frog show receptor sensitivity before functional innervation becomes evident (Higgins and Pappano, 1981; Protas and Leontiva, 1992). If this is also the case for fish, the adrenergic tone in embryonic rainbow trout may be caused by increasing levels of circulating catecholamines and endogenous catecholamines stored within the heart. Some animals show a decrease (Protas and Leontiva, 1992) or no change (Higgins and Pappano, 1981) in β-adrenergic sensitivity once innervation is established.

An increase in heart rate after exposure to atropine indicates a dependence on muscarinic receptors for tonic cardiac regulation (Farrell and Jones, 1992). The cholinergic antagonist atropine had a consistent positive chronotropic effect on the rainbow trout heart after hatching at stage 32, similar to the effect in resting adult rainbow trout (Wood et al., 1979), while the timing of the cholinergic response in the present study is similar to that reported for the golden mullet (Balashov et al., 1991). In larval zebrafish, however, the heart was first responsive to the cholinergic agonist carbachol after hatching at 3 d.p.f. (Hsieh and Liao, 2002) or 4 d.p.f. (Steele et al., 2009), with an established vagal tone at 3 d.p.f. (Hsieh and Liao, 2002; Steele et al., 2009). However, the effect of acetylcholine on heart rate in zebrafish was delayed and did not occur until 5 d.p.f. (Schwerte et al., 2006). Despite the difference between rainbow trout and zebrafish early cardiac physiology, the onset of the heartbeat relative to hatching times is similar. In rainbow trout, a strong heartbeat is first detected at 12 d.p.f. (stage 21), or at approximately 40% of the incubation period from fertilization to hatching (Vernier, 1969; Rombough, 1988a). The zebrafish heart begins to beat at 24 h post-fertilization (hatching occurs at 48–72 h) (Kimmel et al., 1995). In the trout, it is possible that a higher cholinergic tone is gradually acquired in the developmental stages that follow. The lack of a response to cholinergic drugs before stage 32 suggests the absence of cardiac cholinergic receptors or functional pathways. It is not likely that the lack of response is due to saturated cholinergic receptors, as atropine would remove this tone and heart rate would rise. Another explanation may be that acetylcholine stimulates both muscarinic and nicotinic receptors. Nicotinic receptor stimulation of chromaffin cells may have caused the secretion of catecholamines, counteracting a decrease in heart rate by muscarinic stimulation. However, experiments conducted with muscarine (muscarinic receptor-specific agonist, data not shown) produced similar results. Taken together, these results indicate that normal cardiac development occurs in the absence of cholinergic input until at least 2 weeks after hatching.

Early embryos showed an initial rise in heart rate, which remained constant until hatching. Following hatching, the heart rate of larvae decreased. A similar decrease in resting heart rate has been reported for zebrafish following hatching (Bagatto, 2005; Steele et al., 2009). The significant increase in heart rate at stage 26 possibly represents the increased adrenergic tonic stimulation. It is noteworthy that the maximum heart rate at stages 26 and 29 does not coincide with the highest adrenergic tone detected at stages 30 and 32. Possibly, the fall in resting heart rate at stage 30 is related to the initiation of inhibitory vagal tone around this stage, as previously suggested by

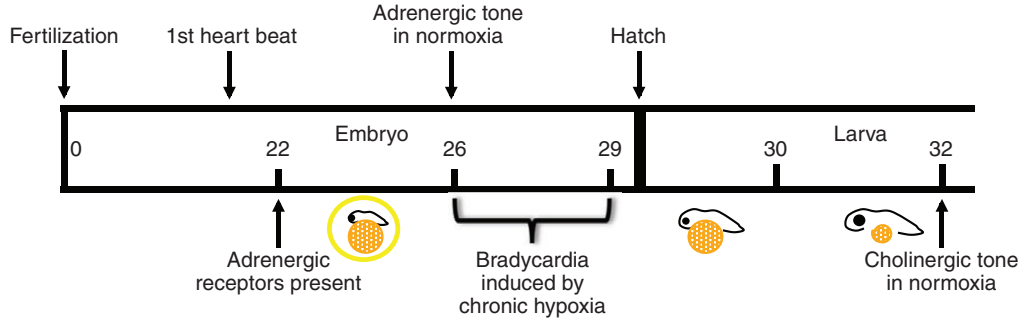

Fig. 7. Adrenergic regulation of heart rate in trout embryos occurs early in development and cholinergic regulation of heart rate is present after hatching. Timeline of the onset of cardiac regulatory mechanisms relative to developmental stage during the early life of rainbow trout (embryonic stages 22, 26 and 29 and larval stages 30 and 32). Embryos were reared from fertilization in normoxia (100% O_2 saturation) or hypoxia (30% O_2 saturation). The development of cholinergic regulation of heart rate in hypoxia-reared embryos was not observed at any of these stages. Developmental stages were designated according to morphological stages (Vernier, 1969).

Holeton (Holeton, 1971). Collectively, in embryos cardio-acceleration reflex mechanisms were in place just after the heartbeat stage, while cardio-inhibitory mechanisms were not functional until after hatching.

The influence of hypoxia exposure on the development of cardiac regulatory control

Chronic hypoxia altered both adrenergic and cholinergic cardiac regulation during the embryonic and larval development of rainbow trout. Interestingly, the onset or activity of adrenergic and cholinergic mechanisms does not simply occur at the programmed developmental stages as in normoxia (Fig. 7). Adrenergic tone was still detected early in development; however, the magnitude of the tone was modified in hypoxia. Surprisingly, the onset of cholinergic receptor responsiveness was completely absent in hypoxia, whereas in normoxia it was present at stage 32. It is evident that the control of heart rate during early life stages depends on environmental O_2.

The comparison between normoxic and hypoxic heart rates over developmental time showed an interesting pattern that may relate to critical developmental events. Initially (stage 22) there was no impact of hypoxia on heart rate; this was followed by a significant decrease (stage 26, 29) and then after hatching the heart rate in hypoxic larvae increased to match that of the normoxic larvae (stage 30, 32). An increase in heart rate in newly hatched individuals relative to embryos in response to the chronic reduction in water oxygen may be necessary to meet the increased metabolic requirements at this stage. Although early larvae meet most of their O_2 requirements by diffusion (Rombough, 1988a), maintaining heart rate and circulation may be essential for delivery of yolk substrates and removal of nitrogenous waste. Interestingly, trout larvae respond to acute hypoxia with a slight tachycardia at least up to 8 days post-hatching (Holeton, 1971). The tachycardia response correlates with the increase in adrenergic scope found in hypoxic larvae after hatching in the present study. In adult rainbow trout, the reverse response is observed during hypoxic exposure, where a slowing of heart rate helps to coordinate blood flow with the supply of oxygen reaching the gills (Farrell, 2007). In larval trout with poorly developed gills, bradycardia would not be of any benefit (Holeton, 1971). It is possible that in chronic hypoxia, the O_2 concentration in the body core of hypoxic embryos is similar to that of larger larvae in normoxia when oxygen diffusion *via* the cutaneous surface can no longer meet the needs of the growing larvae. Therefore, exposure to hypoxia may accelerate the onset of convective oxygen transport. This result also suggests that late stage rainbow trout larvae are capable of a controlled positive chronotropic response from neural or humoral activity.

Hypoxia elicited an increase in adrenergic tone above that found in control embryos as early as stage 26. After hatching, the magnitude of the decrease with propranolol was more profound in hypoxic larvae, and this adrenergic tone progressively increased to reach a maximum at stage 32. In order to maintain heart rate during hypoxia and limit potential inhibition of heart rate by O_2 starvation, it is possible that the imposed decrease in heart rate by limiting O_2 levels is countered by an increase in adrenergic responsiveness, buffering the depressive effects of hypoxia. This change may offset the influence of hypoxia on cholinergic activity. Previous studies with the larvae of other species have also demonstrated that hypoxia exposure stimulates an increase in adrenergic tone, supporting such a role for this response (Crossley et al., 2003b; Steele et al., 2009). This effect has also been shown in adult trout, where adrenergic stimulation of the heart can counteract the negative chronotropic effects of hypoxia (Hanson et al., 2006). Although oxygen is limiting, an increase in β-adrenergic sensitivity likely increases the energy requirements of the heart. This suggests that increased cardiac output is critical for continued development under low O_2 conditions.

The maintenance of heart rate through positive chronotropy is consistent with the hypothesis that the heartbeat is necessary for cardiac morphogenesis and angiogenesis (Burggren, 2004). Potential mechanisms underlying the increased β-adrenergic sensitivity may be found downstream of the receptor and include an increase in the efficiency of energy-producing pathways such as shift to a more efficient isoform of adenylate cyclase or an increased expression of stimulatory G-protein (G_s) rather than inhibitory G-protein (G_i) (Slotkin et al., 2003). In the trout embryos and larvae, the ability of the heart to perform in chronic hypoxia can be due to an increase in pacemaker self-excitation rate by direct adrenergic stimulation of pacemaker cells (Tibbits et al., 1992). In addition, increased adrenergic stimulation allows a β-adrenergic receptor-mediated myocardial Ca^{2+} influx (Vornanen, 1998). This conclusion is supported by the result that blockade of β-receptors under control or optimal oxygenation did not reveal a high adrenergic tone compared with β-adrenergic receptor blockade with hypoxia. Such a response is also present in hypoxic mouse embryos, in which β-adrenergic receptor blockade induced a deeper bradycardia than hypoxia alone, showing that heart rate following β-adrenergic receptor blockade is catecholamine deficient (Portbury et al., 2003). Increased sensitivity to cardiac β-adrenergic receptor stimulation is also seen with hypoxia exposure of fetal sheep (*Ovis aries*) (Parer, 1983) and embryonic chickens (Lindgren and Altimiras, 2009).

Development in hypoxia delayed the appearance of a positive chronotropic response to atropine relative to its appearance in normoxia at stage 32. This suggests that long-term oxygen starvation results in relatively less reliance on the development of cholinergic tone. As vagal function is lacking until after hatching, the hypoxic bradycardia in embryos (stage 26, 29) is not mediated by vagal tone, unlike in adults. This is confirmed by the inability of atropine to abolish the bradycardia. In contrast to the trout, the development of hypoxic bradycardia in zebrafish larvae in response to chronic hypoxia is mediated by cardiac muscarinic receptors (Steele et al., 2009). Similarly, atropine limited the hypoxic bradycardia in late incubation chicken embryos (day 21) but not in younger embryos (days 12–20), indicating that muscarinic receptors play a role in the cardiac response at this stage (Crossley et al., 2003b). The results suggest that environmental insults, such as hypoxia, favor an increase in heart rate in later embryonic stages for survival. Also, in addition to the intrinsic pacemaker rate, there must be scope for positive regulation. Overall, the developmental onset of sympathetic or parasympathetic activity may depend on the stability of environmental conditions in the habitat where development occurs.

Conclusions and perspective

The results of this study illustrate the fact that the adrenergic system is the most potent regulator of heart rate in salmonid embryos. In normoxia, embryos and larvae demonstrated developmental stage-dependent heart rates corresponding to β-adrenergic and cholinergic tone. However, chronic environmental hypoxia altered the normoxic pattern of heart rate regulation by significantly altering the balance and onset of adrenergic and cholinergic cardiac input seen in normoxia. These results demonstrate, therefore, that the ontogeny of cardiac regulatory pathways in slow developing rainbow trout embryos is sensitive to hypoxic conditions and this effect has the

potential to impact the physiological capability of these fish in their native environments.

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