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Environmental modulation of the onset of air breathing and survival of *Betta splendens* and *Trichopodus trichopterus*

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The effect of hypoxia on air-breathing onset and survival was determined in larvae of the air-breathing fishes, the three spot gourami *Trichopodus trichopterus* and the Siamese fighting fish *Betta splendens*. Larvae were exposed continuously or intermittently (12 h nightly) to an oxygen partial pressure (PO_2) of 20, 17 and 14 kPa from 1 to 40 days post-fertilization (dpf). Survival and onset of air breathing were measured daily. Continuous normoxic conditions produced a larval survival rate of 65–75% for *B. splendens* and 15–30% for *T. trichopterus*, but all larvae of both species died at 9 dpf in continuous hypoxia conditions. Larvae under intermittent (nocturnal) hypoxia showed a 15% elevated survival rate in both species. The same conditions altered the onset of air breathing, advancing onset by 4 days in *B. splendens* and delaying onset by 9 days in *T. trichopterus*. These interspecific differences were attributed to air-breathing characteristics: *B. splendens* was a non-obligatory air breather after 36 dpf, whereas *T. trichopterus* was an obligatory air breather after 32 dpf.

Key words: developmental plasticity; heterokairy; hypoxia.

INTRODUCTION

Phenotypic plasticity is the ability of an individual organism to express two or more genetically controlled phenotypes in different environments (Hill *et al.*, 2008). In a developmental context, phenotypic plasticity is evident in developmental plasticity and refers to the fact that there are some susceptible windows of time during ontogeny when an organism's developmental trajectory is altered in response to environmental factors (Pigliucci, 1998; Burggren & Reyna, 2011). During development, this environmentally sensitive phenotype, not the genotype or the gene, is subject to selection and produces individuals with differential fitness (West-Eberhard, 2005).

Phenotypes subject to modification include physiological phenotypes, where a physiological feature of an individual results from specific gene expression and non-genetic regulation for a particular environment (Hochachka *et al.*, 1998). When a physiological feature is expressed during development, it is termed a physiological

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developmental phenotype; variation in its expression is also dependent on environmental variation and this developmental physiological feature affects individual fitness (West-Eberhard, 2005). In other words, a single developing individual is likely to present different physiological phenotypes in different environments, an example of developmental plasticity.

Developmental plasticity can be interpreted in the framework of two concepts: heterochrony and heterokairy. Heterochrony is the altered timing of the developmental events as a mechanism of evolution (Gould, 1977). Specifically, heterochrony is the genetically based alterations in development (as evident from different times of appearance of developmental landmarks) between different species (Spicer *et al.*, 2011). Physiological heterochrony, then, is the alteration of the timing in the development (first appearance or subsequent maturation) of physiological traits in one species relative to an ancestral species (Spicer & Rundle, 2006).

Heterochrony is useful in understanding developmental changes at the species level but, importantly, such changes also occur at the population and individual levels and drive the composition of the gene pool. Consequently, to understand developmental plasticity at that level, the concept of heterokairy was introduced (Spicer & Burggren, 2003; *Spicer et al*., 2011). Heterokairy is the non-genetic change in the timing of the onset of physiological regulatory systems, or plasticity in the timing of the onset of physiological regulatory systems or their components, below the level of the species, between populations or individuals.

Physiological heterokairy is a relatively new concept and as such does not have a great deal of direct experimental support, particularly in vertebrates. The concept has been gaining credibility based on the reinterpretation of previous data collected to show developmental plasticity. In an analysis by Spicer & Rundle (2007), heterokairy at the individual level was considered, and they summarized empirical evidence of this phenomenon. For example, the development of salinity tolerance in salmonids during the transition from fresh water to a marine existence was brought forward in development (*i.e*. occurred earlier) when brook trout *Salvelinus fontinalis* (Mitchill 1814) were pre-exposed to sea water (Hiroi & McCormick, 1995), coho salmon *Oncorhynchus kisutch* (Walbaum 1792) were administered cortisol or insulin (McCormick *et al.*, 1991; McCormick, 1995) or Atlantic salmon *Salmo salar* L. 1758 were exposed to temperature treatments (Handelanda *et al.*, 2004). Other examples involve the Atlantic herring *Clupea harengus* L.1758, where the thermal environment alters the ontogeny of muscle physiology, such that the rostral-to-caudal progression of myofibril synthesis as well as slow fibre innervation appears earlier as environmental temperature increased (Johnston *et al.*, 1997).

Specific developmental markers for heterokairy were affected by temperature in haddock *Melanogrammus aeglefinus* (L. 1758) (Martell *et al.*, 2006). Blastopore closure, notochord vacuolation, retinal pigmentation, the appearance of blood cells and hatching occurred later in development at lower temperatures. Appearance of the optic lumen, neural tube cavitation and increased myofibril density per deep cell occurred earlier at lower temperatures. Notochord and eye development were accelerated with increased embryonic temperature, whereas myofibrillar genesis and neural tube development were similarly slowed.

As a final example, California grunion *Leuresthes tenius* (Ayres 1860) provides evidence for heterokairy involving plasticity in the hatching time. Embryos from

the same spawning hatched at different times and different developmental stages (Moravek & Martin, 2011). They responded to an unpredictable environment with diapause and then responded rapidly by hatching when the environment became favourable.

Studies of heterokairy involving physiological processes such as respiration and metabolism in vertebrates, including fishes, are lacking. The onset of aerial respiration in air-breathing fishes in the context of heterokairy was investigated here. Air breathing in bimodal breathing fishes and amphibians is the culmination of the maturation of an entire suite of structures and processes, ventilatory control mechanisms, effective blood perfusion pathways and specialized air–blood interfaces that create a functional air-breathing organ (ABO) (Burggren & Warburton, 2007). Air breathing is also a trait that is likely to confer high fitness on aquatic larval fishes exposed to aquatic hypoxia, and so hypoxia-driven natural selection of a development plan for air breathing that is modifiable might be anticipated. Air breathing as a behaviour in labyrinth fishes can be easily tracked because it requires surfacing and highly stereotypic body movements and is further signalled by the release of a gas bubble from the suprabranchial chamber containing the labyrinth organ. The onset of air-breathing behaviour is also easy to follow by visual observation or with video devices (Blank, 2009; Blank & Burggren, 2014). These characteristics make the onset of air breathing an ideal model for studying heterokairy related to aerial gas exchange.

The specific purpose of this study was, then, to determine if chronic hypoxia could promote developmental plasticity in the onset of air-breathing fish larvae, comprising the first vertebrate experiment designed to test the heterokairy hypothesis with a discrete physiologically related developmental marker. Two easily reared and handled species of labyrinth air-breathing fishes readily lend themselves to the testing of the heterokairy hypothesis in the laboratory. The three spot gourami *Trichopodus trichopterus* (Pallas 1770) is an obligate air breather (Burggren, 1979; Graham, 1997; Herbert & Wells, 2001; Blank, 2009; Burggren & Blank, 2009) and the Siamese fighting fish *Betta splendens* Regan 1910 is a non-obligatory air breather (Peters, 1978; Graham, 1997).

MATERIALS AND METHODS

FISH SPECIES USED IN EXPERIMENTATION

Experiments were conducted on two different labyrinth fishes from the Osphronemidae: *T. trichopterus* subfamily Luciocephalinae and *B. splendens* subfamily Macropodusinae (Froese & Pauly, 2013). Both species develop rapidly and they produce large numbers of eggs in each laying: 500 and 1000–2000, and these eggs hatch within 24–48 h. Their fry become free-swimming in 3–4 days (at 28° C) (Pollak, 1981).

ANIMAL MAINTENANCE AND BREEDING

A total of 10 breeding pairs of the *T. trichopterus* were obtained from the reproductive stock maintained in the Developmental Integrative Biology Laboratory of the University of North Texas. A total of 10 breeding pairs of *B. splendens* were obtained from the Carolina Biological Supply Company (www.carolina.com). Both species were maintained and conditioned for breeding in 401 aquariums equipped with under-gravel filters, held at 27°

C and a 12L:12D photoperiod. Water chemistry was maintained as follows: 6·5–7·5 pH, $<40\,mg\,l^{-1}$ nitrate, $<0{\cdot}5\,mg\,l^{-1}$ nitrite, $50–120\,mg\,l^{-1}$ total hardness and 20 kPa of oxygen partial pressure (PO_2). Adult fishes and breeders were fed *ad libitum* twice per day with a high protein diet (>40%) of dry flakes and frozen brine shrimp *Artemia* sp. and bloodworms *Chironomus* sp.

Separate male and female groups comprising four to six adult *T. trichopterus* were isolated for 2–3 weeks at 28° C. Once a male showed darkened fins, reproduction was induced by moving into an aquarium at 30–32° C for 1 week or until it started to build a visible bubble nest. A female showing a bulky abdomen was then placed with the primed male and nocturnal spawning typically occurred within the next 2 days.

Two groups of adult *B. splendens*, one of four males and the other of four females, were isolated for 2–3 weeks at 28° C. Individuals were isolated in the same aquarium by a divider due to their aggressiveness. Once a male showed bubble nest building behaviour, a female showing bulky abdomen and a white spot on the vent was placed next to the male in a floating fine mesh cage. If the courtship dance occurred between them, the female was released from the cage and nocturnal spawning typically occurred within the next 2 days. After spawning, the female was taken out to avoid attacks from the male.

LARVAL STAGING AND HANDLING

All the eggs for the same experiment were taken from the same clutch to avoid intra-clutch effects. The chronological time was registered as standardized age in days post-fertilization (dpf). The fertilization day was considered day 0, and for every cycle of 24 h, a unit of day was added. The experiment stopped at 50 dpf.

After 12 h of fertilization, eggs produced from the single clutch were removed from the male's bubble nest and placed in 20 l experimental tanks. Each tank was equipped with a sponge biofilter and was maintained at 27° C with a 12L:12D photoperiod. The water quality for larvae was evaluated at shorter intervals than that of water used for breeding and was maintained as follows: 7·3–7·5 pH, $<10\,mg\,l^{-1}$ nitrate, $<0{\cdot}5\,mg\,l^{-1}$ nitrite and $80–100\,mg\,l^{-1}$ total hardness.

The larvae of both species completely consumed their yolk around 5 dpf. After that time, they were fed *ad libitum* with live microworms *Panagrellus redivivus* from days 6 to 10. Thereafter, they were fed with live *Artemia* sp. newborn nauplii. Prior to all measurements, larvae were deprived of food for 12 h. They were then moved to experimental cages in aquaria containing water with the same water quality and allowed to acclimate for *c.* 1·5 h before experimentation. The experimental cages consisted of floating plastic circular cages with a <0·2 mm mesh placed in 20 l aquaria maintained at a specific oxygen level. Oxygen levels in the aquaria containing the experimental chamber were regulated by bubbling into the water either room air (20 kPa) or a mixture of room air and pure nitrogen, to achieve one of the two desired levels of hypoxia (17 or 14 kPa). Each tank was filled with conditioned water to four fifths of its capacity, with the remaining one fifth filled with the gas that bubbled up through the aquarium. The tank was sealed with a plexiglass cover with a valve to prevent atmospheric air from leaking back into the tanks and to ensure that the gas and water phases were in PO_2 equilibrium. Water PO_2 was monitored daily with an optical oximeter probe ProODO (YSI Incorporated; www.ysi.com). The PO_2 of the gas above the water was measured and monitored daily with a ProOx 110 oxygen sensor (Biospherix Ltd; www.biospherix.com). Exposing the experimental larvae to hypoxic air was not the natural condition for them, but it was deemed necessary for this study to ensure that the larvae could not escape internal tissue hypoxia through respiration with air.

Two types of hypoxic exposure, intermittent and continuous, were created in different experiments. Nocturnal intermittent exposure mimicked the dial cycle of hypoxia in tropical habitats where these fishes have evolved, namely 12 h of normoxia during the day and 12 h of hypoxia during the night. That is, hypoxic exposure was synchronized with the photoperiod employed.

Each PO_2 population was created in triplicate, with 150 larvae placed in three cages (50 on each) to develop and grow from 0 to 50 dpf. Water in the aquaria and within the experimental cages was continuously circulated using a submersible pump (Marineland Maxy-jet 400;

www.marineland.com) to maintain homogeneous conditions of PO_2 and water quality within the cages.

MEASUREMENT OF LARVAL SURVIVAL

Larval mortality for each day was registered for each of the three cages. The proportion of survivors (S) was determined as $S = (N_i - N_{CD})\, N_0^{-1}$, where $N_0 =$ initial number of larvae in the cage ($n = 50$) and $N_{CD} =$ cumulative number of dead larvae per day. Survival was compared between the three PO_2 levels and between continuous and intermittent exposure.

MEASUREMENT OF ONSET OF AIR BREATHING

Air breathing was measured in a group of five larvae from each PO_2 treatment with two replicate measurements for each group. A different group of larvae was randomly selected daily. Fishes that were used for experimentation were removed from the general pool and not used again. This group of larvae was exposed to extreme aquatic hypoxia (PO_2: 3–5 kPa) for 10 min to see if air breathing was stimulated. A fish was considered to be air breathing when it surfaced to inhale air and subsequently released a bubble of gas through the opercular opening. The percentage of air breathers per day was recorded from 15 dpf until 100% of the sample had begun breathing air.

STATISTICAL ANALYSIS

The frequencies of survival and onset of air-breathing independence of PO_2 were tested by arranging contingency tables with treatment in the columns and age in the rows and using a χ^2 analysis (Zar, 2010). Additionally, a Kruskal–Wallis (H) comparison was performed within the onset of air breathing of each PO_2 level. The onset of air breathing for each larva was computed in dpf. Additional Spearman rank correlation (r_s) was used to identify patterns between the onset of air breathing and levels of PO_2.

RESULTS

LARVAL SURVIVAL

Betta splendens in continuous normoxic conditions during the 40 dpf period had a survival rate of 65–75% to 50 dpf. The corresponding value for *T. trichopterus* larvae was far lower, at 15–30% [Figs 1(a) and 2(a)]. For *B. splendens*, survival depended on the PO_2 treatment for both continuous exposure [$\chi^2 = 575$, d.f. $= 74$, $P < 0{\cdot}001$; Fig. 1(a)] and nocturnal intermittent exposure [$\chi^2 = 207$, d.f. $= 74$, $P < 0{\cdot}001$; Fig. 1(b)]. The highest survival rate was in continuous normoxia (>65%), whereas nocturnal intermittent hypoxia produced a low survival rate [<25%; Fig.1(b)]. No larvae survived continuous hypoxia beyond 9 dpf [Fig. 1(a)]. For *T. trichopterus*, survival depended on the PO_2 treatment for both continuous exposure ($\chi^2 = 2830$, d.f. $= 60$, $P < 0{\cdot}001$) and nocturnal intermittent exposure [$\chi^2 = 175$, d.f. $= 72$, $P < 0{\cdot}001$; Fig. 2(a), (b)]. Survival rate in nocturnal intermittent hypoxia was not significantly different from continuous normoxia [Fig. 2(b)], but no larvae survived continuous hypoxia beyond 9 dpf [Fig. 2(a)].

Collectively, these initial findings of survival showed that continuous hypoxia was deleterious for larvae to 9 dpf. Consequently, the remainder of experiments were conducted either under nocturnal intermittent hypoxia conditions or, for controls, continuous normoxia.

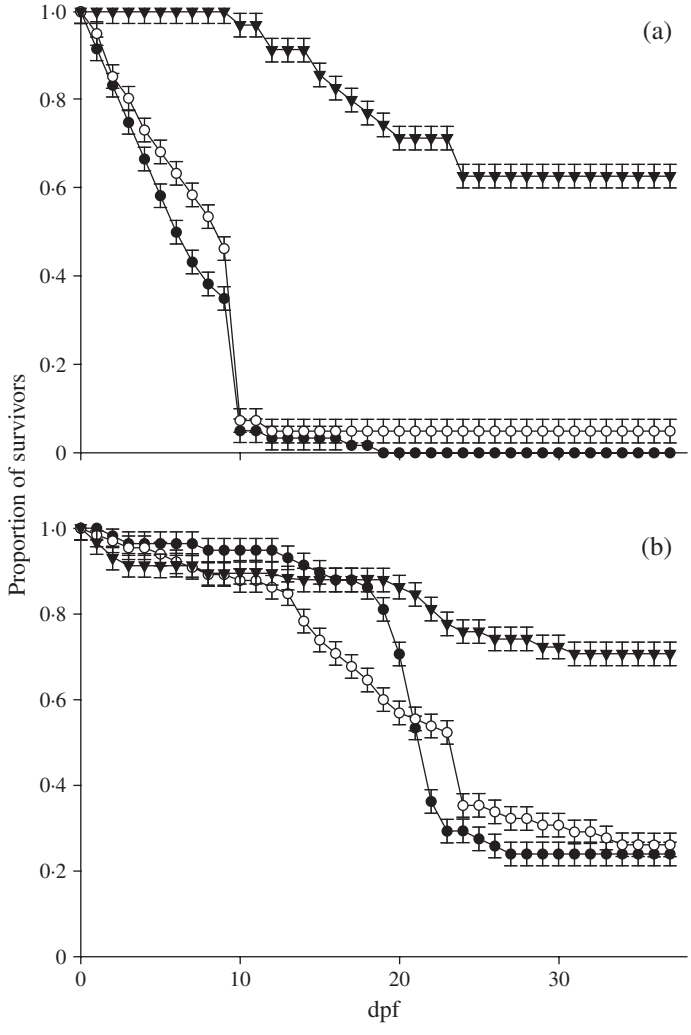

FIG. 1. Survival of *Betta splendens* (days post-fertilization, dpf) in three water PO_2 treatments: (a) continuous hypoxia and (b) intermittent hypoxia (▼, 20 kPa; ○, 17 kPa; ●, 14 kPa). Values are mean ± S.E.

AIR-BREATHING ONSET

In a preliminary experiment with three normoxic groups of larval *B. splendens* from different clutches and parents, the onset of air breathing was significantly different between clutches ($H = 74$, $n_1 = 22$, $n_2 = 31$, $n_3 = 45$, $P < 0{\cdot}001$) with average and range values (in dpf) of 26 (25–27), 26 (23–29) and 40 (37–42). To avoid intra-clutch variation, the survival and onset of air-breathing experiments were therefore conducted with larvae from the same clutch.

The air-breathing onset for *B. splendens* in continuous normoxic exposure was an average of 39 dpf within a range of 37–42 dpf, with 50% of juveniles breathing air at 38 dpf [Fig. 3(a), (b)]. In contrast, the average onset of air breathing at continuous

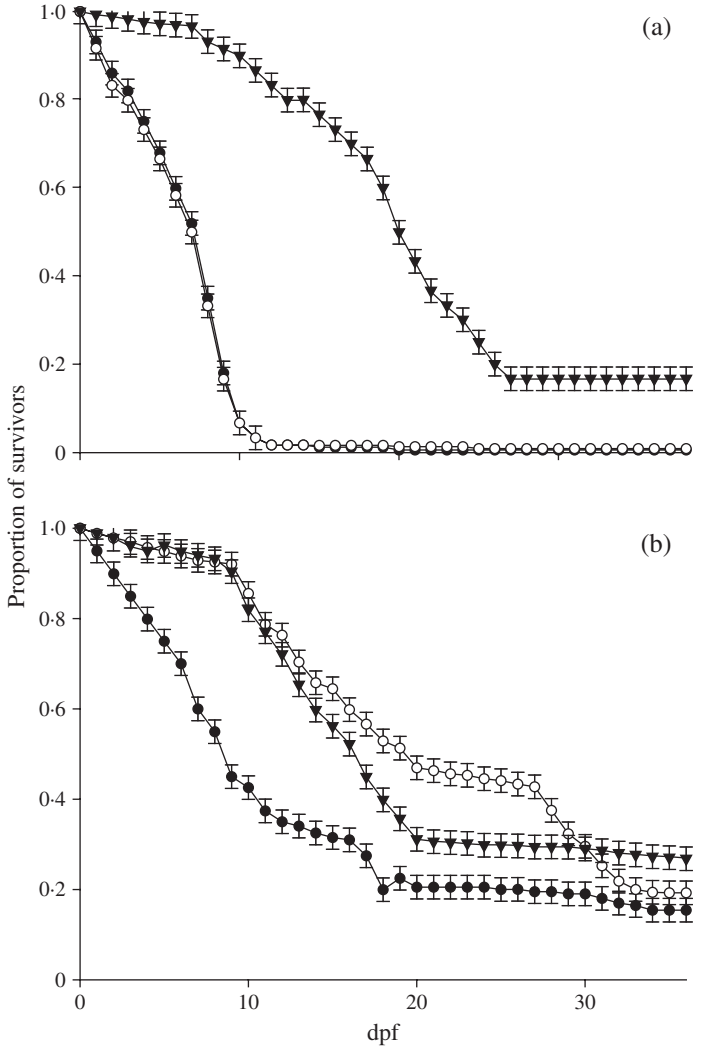

FIG. 2. Survival of *Trichopodus trichopterus* (days post-fertilization, dpf) in three water PO_2 treatments: (a) continuous hypoxia and (b) intermittent hypoxia (▼, 20 kPa; ○, 17 kPa; ●, 14 kPa). Values are mean ± S.E.

normoxic exposure in the *T. trichopterus* was 35 dpf within a range of 32–37, with 50% of larvae breathing air at 34 dpf [Fig. 4(a), (b)].

To test the hypothesis that the time of onset of air breathing could be modulated by hypoxia exposure in both *B. splendens* and *T. trichopterus*, larvae of each species drawn from the same clutch were exposed to one of continuous normoxia and two levels of nocturnal intermittent hypoxia (17 and 14 kPa). For *B. splendens*, the onset of air breathing depended on PO_2 treatment ($\chi^2 = 56$, d.f. $= 24$, $P < 0{\cdot}001$). Nocturnal intermittent hypoxia at 17 kPa advanced the onset of air breathing by 4 days [Fig. 3(b)]. Moreover, the entire sample of the 14 kPa PO_2 group began air breathing 2 days before the normoxic group [Fig. 3(a)]. These results suggest

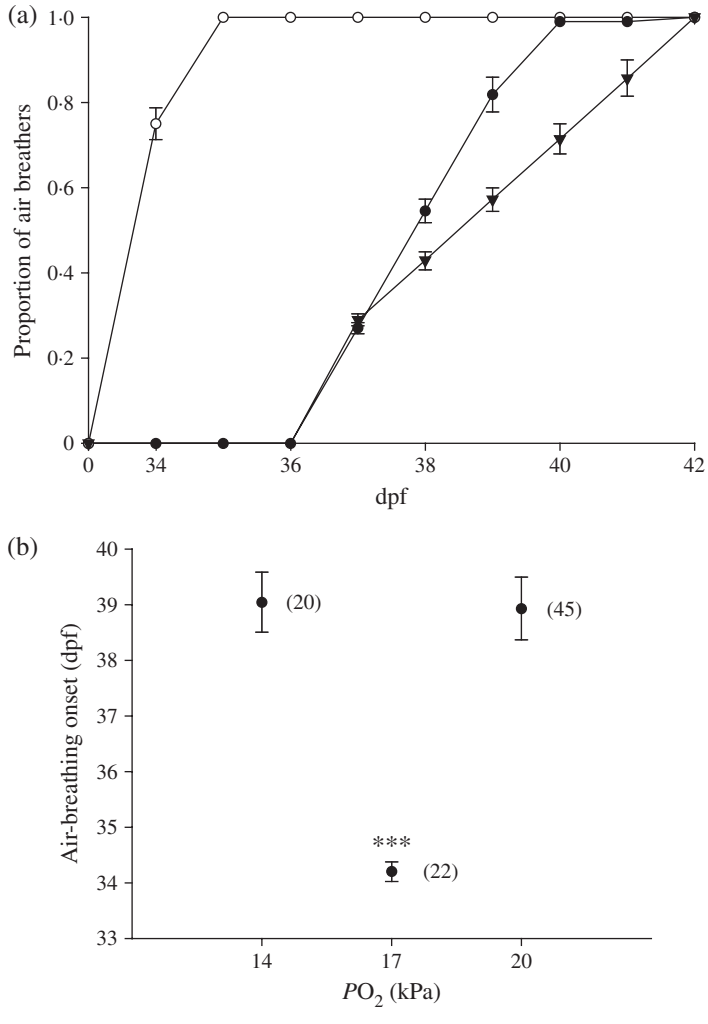

FIG. 3. (a) Proportion of *Betta splendens* larval air breathers through development (days post-fertilization, dpf) at different partial pressure of oxygen (PO_2) treatments (▼, 20 kPa; ○, 17 kPa; ●, 14 kPa). Values are mean ± S.E. (b) Onset of air breathing by PO_2 treatment. Values are means ± 95% C.I. [sample sizes are in parenthesis; *, significant differences ($P < 0{\cdot}001$)].

that nocturnal hypoxia draws forward the onset of air breading [$H = 50$, $n_{21} = 45$, $n_{18} = 22$, $n_{15} = 20$, $P < 0{\cdot}001$; Fig. 3(b)].

In contrast to *B. splendens*, the onset of air breathing in *T. trichopterus* was independent of PO_2 treatment [$\chi^2 = 9{\cdot}3$, d.f. $= 26$, $P > 0{\cdot}05$; Fig. 4(a), (b)]. In a non-parametric comparison, however, the air-breathing onset showed significant differences and the total sample of hypoxic groups advanced to the air-breathing stage 8 days later than the normoxic group ($H = 26{\cdot}0$, $n_{21} = 40$, $n_{18} = 32$, $n_{15} = 25$, $P < 0{\cdot}001$). These results suggest that intermittent, nocturnal hypoxia delays the onset of air breathing in at least 60% of the population [Fig. 4(b)].

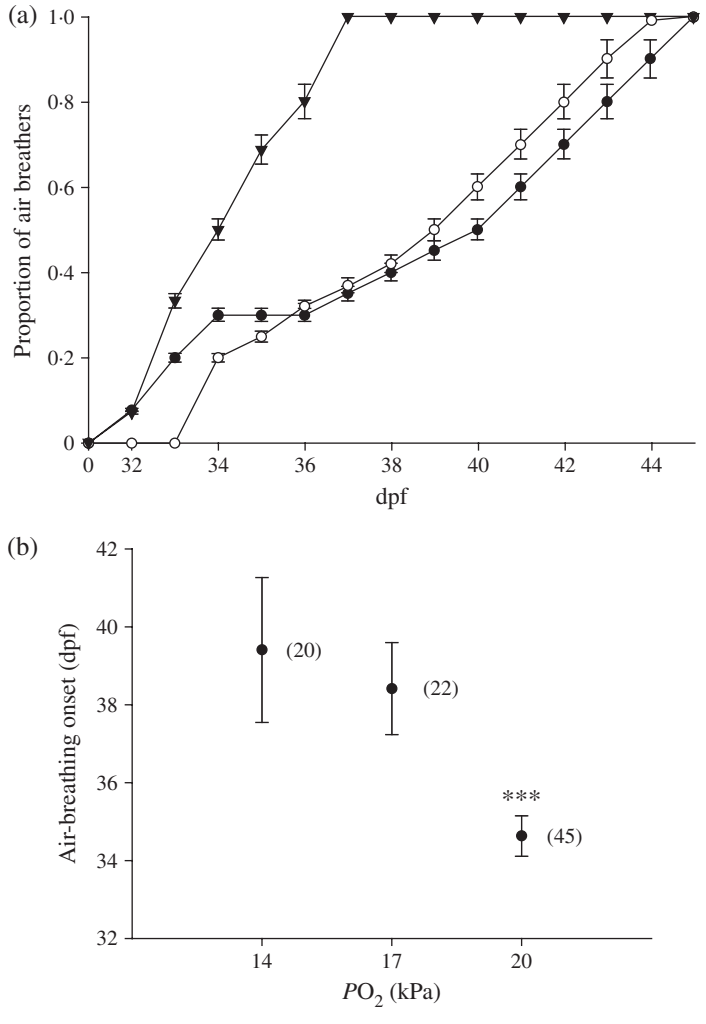

FIG. 4. (a) Proportion of *Trichopodus trichopterus* larval air breathers through development (days post-fertilization, dpf) at different partial pressure of oxygen (PO_2) treatments (▼, 20 kPa; ○, 17 kPa; ●, 14 kPa). Values are mean ± S.E. (b) Onset of air-breathing by PO_2 treatment. Values are mean ± 95% C.I. [sample sizes are in parenthesis; *, significant differences ($P < 0{\cdot}001$)].

DISCUSSION

LARVAL SURVIVAL RATES

Survival of a fish under hypoxic conditions depends on several basic capabilities. Of primary importance is the ability to increase ventilation of the gas exchange organs, accompanied by appropriate changes in blood perfusion of these organs (Burggren & Warburton, 2007). Failing this adjustment, of importance is the capacity to decrease overall metabolic rate (metabolic depression), to tolerate increased levels of metabolic by-products (particularly protons produced by anaerobic metabolism

and associated with lactic acid accumulation) and to avoid and repair cellular injury following reoxygenation (Bickler & Buck, 2007). Fish larvae are early in the process of developing the systems necessary to respond or to tolerate hypoxia. When combined with the fact that hypoxic stress alters the overall developmental programming (Burggren & Bagatto, 2008), hypoxia can generate mass larval fish mortalities in natural environments (Houde, 2002; Diaz & Breitburg, 2009).

This study revealed differences in larval survival between continuous and intermittent hypoxia treatments for both *B. splendens* and *T. trichopterus.* Continuous hypoxic stress probably overwhelmed the response and tolerance capabilities of the larvae, leading to their death. The result of this was complete population mortality in continuous hypoxia by 9 dpf for both species. Nocturnal intermittent hypoxia, on the other hand, probably gave larvae sufficient time to recover and still continue subsequent development during alternate episodes of stress, recovery and normal development. Interestingly, this study found that *T. trichopterus* was slightly more sensitive to hypoxia in early larval life than reported for this species in an independent study (Blank & Burggren, 2014). There are numerous reasons for physiological variation between studies, and for fish species, the susceptibility of larvae to hypoxia can be altered by epigenetic effects relating to maternal and paternal experiences (Ho & Burggren, 2010; Burggren, 2014).

Mean ± S.E. natural initial mortality rate for freshwater larval fishes has been estimated to be as high as 16 ± 4% per day (Fuiman, 2002; Houde, 2002). In this experiment, the mean ± S.E. mortality per day during the experimental period (40 dpf) in the continuous normoxia (20 kPa) was 1·4 ± 0·1% for *B. Splendens* and 2·1 ± 0·1% for *T. trichopterus*; the same parameter in continuous hypoxia was 2·7 ± 0·3 and 2·7 ± 0·2%, and in nocturnal intermittent hypoxia was 2 ± 0·1 and 2·2 ± 0·06% for each species. In this case, the daily hypoxic-induced mortality was <2·7% of the natural per-day mortality in all cases. This high survival rate can probably be explained by the absence of predation, abundant food supply and the maintenance of high water quality in a laboratory setting.

The absolute expected mortality of larval freshwater fishes is *c.* 95% in natural habitats (Fuiman, 2002; Houde, 2002). In this study, mortality in response to continuous hypoxia was close to 100% for both species. In contrast, mortality under intermittent, nocturnal treatment was 74–76% for *B. splendens* and 81–85% for *T. trichopterus.* Given these higher survival rates, intermittent hypoxia treatment was subsequently employed to simulate the natural diel oxygen cycle where these two species of air-breathing fishes evolved, namely tropical freshwater ponds with high autotrophic oxygen production during the day and high heterotrophic oxygen demand during the night (Graham, 1997; Farrell & Richards, 2009; Verberk *et al.*, 2011). Although more difficult to produce technically, clearly intermittent hypoxia creates a more natural environmental stimulus and is recommended for future experimental exposure to hypoxia.

These results highlight dissolved oxygen as a limiting environmental factor for larval fishes in fresh water (Diaz & Breitburg, 2009), and hypoxia as a strong natural selection force as in adult air-breathing fishes (Randall *et al.*, 1981; Graham, 1997) with a high cost evident in lowered larval survival. The current findings also showed that the response of *B. splendens* to hypoxia is not the same as in *T. trichopterus* (Farrell & Richards, 2009). Possible reasons for these differences are discussed below.

HYPOXIC MANIPULATION OF AIR-BREATHING ONSET

The air-breathing onset for anabantids has been reported to take place over the broad species-specific range of 18–29 dpf (Prasad & Prasad, 1985; Graham, 1997) and specifically for *T. trichopterus* at 20–25 dpf (Blank, 2009). Under normoxic conditions in this study, air-breathing onset occurred at 37–40 dpf for *B. splendens* and at 32–36 dpf for *T. trichopterus.* The difference from previously reported data for anabantids suggests a high variation of this variable as a result of heterochrony. In *B. splendens*, there was a considerable inter-clutch variation in the onset of air breathing. Variation in the chronological age (timing) when normal air-breathing onset occurs reflects developmental plasticity (West-Eberhard, 2005), as well as different developmental trajectories for each species and individuals within populations (Burggren & Reyna, 2011).

This study shows that hypoxic exposure can alter the timing of air-breathing onset within species. As such, these data support the theory of heterokairy, in which developmental timing of specific physiological process such as aerial respiration has been moved as a result of an environmental stressor such as hypoxia (Spicer & Burggren, 2003; Spicer *et al.*, 2011). The results showed a particular pattern for each species. In *T. trichopterus*, PO_2 of both 17 and 14 kPa delayed the onset of air breathing in an inversely correlated way ($r_s = -1$, d.f. $= 1, 2$, $P < 0{\cdot}001$). In contrast, intermittent hypoxia at a level of 17 kPa of PO_2 brought air breathing forward in the developmental plan for *B. splendens* but hypoxia at the lower level of 14 kPa had no significant effect. The reason for this differential response is not evident, but the dose–response curve for the oxygen effect on the developmental plan should not be assumed to be a simple linear relationship. Indeed, at lower oxygen levels, larvae may simply not be able to mount an adaptive response. The interspecific differences between *B. splendens* and *T. trichopterus* in the developmental trajectory (Burggren & Reyna, 2011) can be considered evidence of heterochrony (Gould, 1977; Spicer *et al.*, 2011) because both species diverge from the same phylogenetic family, Osphronemidae (Rüber *et al.*, 2006; Froese & Pauly, 2013).

These interspecific differences in hypoxic effects may be related to ecophysiological differences between species. Obligatory air-breathing forms, such as *T. trichopterus*, are unable to survive on the quantity of O_2 obtained by purely aquatic respiration (branchial and cutaneous), even in normoxic water, and thus they always need supplemental aerial oxygen. By contrast, non-obligatory air breathers including *B. splendens* do not require air breathing to survive in normoxic water but can survive on continuous aquatic respiration (Lefevre *et al.*, 2014).

Based on the previous descriptions, the onset of air breathing in these Osphronemidae species proved to be a discrete developmental marker for testing the hypothesis of heterokairy. The present experiments show that *B. splendens* is a non-obligatory air breather after 36 dpf and hypoxia accelerates its air-breathing onset, while *T. trichopterus* is an obligatory air breather past 32 dpf and hypoxia delays its onset of air breathing. Both findings support the hypothesis of physiological heterokairy.

Because the onset of air breathing can be accelerated or delayed by hypoxia, the onset of air breathing, which involves numerous physiological regulatory processes, represents a clear example of the phenomenon of heterokairy (Spicer & Burggren, 2003). Heterokairy serves as a form of compensation to environmental stressors such as oxygen. Although examples within vertebrates of heterokairy are as yet few in number, the movement in development of the appearance of the ABO and its

ventilation represents a case of heterokairy that involves multiple levels of regulation. Modifications must occur in numerous morphological structures and a whole series of physiological regulatory systems that involve these structures (*e.g.* respiratory, cardiovascular and musculoskeletal). In the case of fishes exposed to hypoxia, known responses can include cellular-molecular, physiological, anatomical and behavioural changes (Diaz & Breitburg, 2009), and it might be reasonably anticipated that these responses can additionally involve heterokairy.

Unknown at this time is the ultimate cost of responses representing heterokairy in animals. By way of comparison, the thrifty phenotype sometimes evident in very low birth mass mammal infants is helpful in infancy and in regaining normal body mass in juveniles but ultimately proves maladaptive later in adult life when the metabolic syndrome appears (Barker, 1997; Isezuo, 2006). Whether heterokairic responses in larvae that aid larval survival ultimately prove maladaptive awaits further study.

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