Improving sneaky-sex in a low oxygen environment: reproductive and physiological responses of male mosquito fish to chronic hypoxia

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Summary

Few studies have examined the adaptive significance of reversible acclimation responses. The aerobic performance and mating behaviour of the sexually coercive male eastern mosquito fish (Gambusia holbrooki) offers an excellent model system for testing the benefits of reversible acclimation responses to mating success. We exposed male mosquito fish to normoxic or hypoxic conditions for 4 weeks and tested their maximum sustained swimming performance and their ability to obtain coercive matings under both normoxic and hypoxic conditions. We predicted that hypoxia-acclimated males would possess greater swimming and mating performance in hypoxic conditions than normoxic-acclimated males, and vice versa when tested in normoxia. Supporting our predictions, we found the sustained swimming performance of male mosquito fish was greater in a hypoxic environment following long-term exposure to low partial pressures of oxygen. However, the benefits of acclimation responses to mating performance were dependent on whether they were tested in the presence or absence of male–male competition. In a non-competitive environment, male mosquito fish acclimated to hypoxic conditions spent a greater amount of time following females and obtained more copulations than normoxic-acclimated males when tested in low partial pressures of oxygen. When males were competed against each other for copulations, we found no influence of long-term exposure to different partial pressures of oxygen on mating behaviour. Thus, despite improvements in the aerobic capacity of male mosquito fish following long-term acclimation to hypoxic conditions, these benefits did not always manifest themselves in improved mating performance. This study represents one of the first experimental tests of the benefits of reversible acclimation responses, and indicates that the ecological significance of physiological plasticity may be more complicated than previously imagined.

Key words: acclimation, phenotypic plasticity, hypoxia, coercive mating, physiological plasticity.

Introduction

Many freshwater environments have extreme temporal and/or spatial variation in the partial pressure of dissolved oxygen. The effective uptake of oxygen from aquatic environments is critical to the survival of all fishes and influences changes in their physiology, behaviour and distribution. Fish possess a range of rapid behavioural and physiological responses that can minimise the costs associated with decreases in the partial pressure of oxygen by allowing either more effective uptake of oxygen or decreasing their requirements. These include increases in the opercular beat rate (Maxime et al., 2000), changes in plasma pH causing a left shift in the haemoglobin–oxygen binding affinity (Nikinmaa and Salama, 1969), aquatic surface respiration (ASR) (Hughs, 1973; Kramer and Mehegan, 1981; Burggren, 1982; Kramer and McClure, 1982; Chapman et al., 1995), behavioural hypothermia (Jensen et al., 1993) and microhabitat selection and movement patterns (Wannamaker and Rice, 2000).

Long-term decreases in the partial pressure of oxygen can also elicit a range of other physiological responses in fish that can allow more effective uptake of oxygen under hypoxic conditions. For example, haematocrit, haemoglobin and erythrocyte concentrations increase in some species after several weeks of exposure to hypoxic conditions (Frey et al., 1998; Timmerman and Chapman, 2004b; Jensen et al., 1993), whereas the oxygen binding affinity can be modified in others (Love and Rees, 2002). In addition, increases in gill surface area have been reported in the sailfin molly (Poecilia latipinna) acclimated to hypoxia (Timmerman and Chapman, 2004a). However, despite the diversity of studies demonstrating the physiological mechanisms underlying improved uptake of oxygen under hypoxic conditions, few studies have addressed the ecological or fitness consequences of short- or long-term changes in the partial pressure of oxygen in the aquatic environment (Kramer and Mehegan, 1981; Hale et al., 2003). This is surprising given the obvious importance of environmental oxygen availability on the evolution of freshwater biota.

Studying the ecological or fitness consequences of
physiological responses is often problematic because of the difficulties associated with linking physiological traits with their direct reproductive or survival benefits (Wilson and Johnston, 2004). Most studies attempting to relate physiological traits to their ecological significance use measures of locomotor performance as a proxy of fitness, because of its presumed connection with escape from predators or prey-capture. However, it is often not clear whether variation in locomotor performance actually influences survival or reproductive success in the wild (Walker et al., 2005).

Linking physiological studies with reproductive behaviour offers an alternative opportunity for examining the direct implications of variation in physiological function with reproductive performance. Behavioural ecologists have already successfully utilised the mating behaviour of organisms to examine the ecological and reproductive consequences of variation in both morphological and behavioural attributes (Condon and Wilson, 2006).

The effect of environmental oxygen on the mating behaviour of male eastern mosquito fish offers an excellent opportunity for examining questions related to the fitness consequences of variation in physiological traits. The mosquito fish is a viviparous poeciliid fish native to the south-eastern USA and is an introduced pest species across much of eastern Australia. Mosquito fish are ideal for studies of acclimation as they inhabit a wide range of freshwater environments from swampy hypoxic conditions to well-oxygenated streams. Mosquito fish have been used extensively as a model system in studies of variation in physiological traits. The mosquito fish is a for examining questions related to the fitness consequences of physiological acclimation.

**Methods and materials**

Eastern mosquito fish *Gambusia holbrooki* Girard were collected from 18 Mile Swamp on North Stradbroke Island, Queensland. Fish were returned to the University of Queensland and maintained in single-sexed groups in 60 l aquaria and fed daily on flaked fish food. After at least 2 weeks in the laboratory, approximately 60 males and 60 females were separated into two treatment groups; hypoxia acclimation and normoxia acclimation (14 h:10 h light:dark). For all experimental tanks, hypoxic conditions were maintained by bubbling commercially purchased N₂ through a single air stone and decreasing any surface air movement by fitting tanks with glass lids. Partial pressure of oxygen for the hypoxic treatment was maintained between 3.1–4.0 kPa (approx. 15–20% saturated) throughout the experimental period. Air was constantly aerated through the normoxic tanks to ensure saturation. The aquaria were maintained at 20°C throughout the acclimation period.

After a 6-week acclimation period, the sustained swimming performance of each individual fish was tested under both normoxic and hypoxic conditions. In addition, the mating behaviour of male mosquito fish was measured under both non-competitive and competitive conditions in both hypoxia and normoxia. For all experimental tests, the first environment tested for each individual (or pair) was selected at random. All testing was conducted at 28°C, which represents a temperature that approximates the optimum for both coercive mating and swimming performance of male mosquito fish (Wilson, 2005).

**Swimming performance**

The sustained swimming performance of each individual male mosquito fish was tested under normoxic and hypoxic conditions (N=15 for hypoxic males; N=15 for normoxic males). Sustainable swimming speeds were measured in a Brett-type swimming flume that consisted of a 10 cm long swimming section of 3 cm diameter through which a continuous water current was delivered by an adjustable motor connected to a rotor. Fish were introduced into the flume at a water velocity of 2 cm s⁻¹ and allowed to settle for 5 min. The velocity of the current within the flume was then increased by 2 cm s⁻¹ every 3 min until the fish showed signs of fatigue. Given the short period between increases in water velocity, the underlying physiological basis for these measures of swimming performance are most likely aerobic and anaerobic in nature. A fish was defined as fatigued when it could no longer hold its position and was swept against the back grid. The total time to exhaustion and the water velocity at exhaustion were recorded for each fish and used to calculate the sustained swimming performance \( U_{\text{max}} \) with the equation (Brett, 1964):

\[
U_{\text{max}} = U_i + \left[ \left( T_i/T_f \right) U_i \right],
\]

where \( U_i \) is the highest speed maintained for an entire 3 min interval, \( T_i \) is the time taken to exhaustion in the final speed interval, \( T_f \) is the time interval length (3 min), and \( U_i \) is the speed increment (2 cm s⁻¹) [modified from Brett (Brett, 1964)].
Mating behaviour

Mating performance of the males was assessed in a non-competitive and male–male competitive environment. For the non-competitive tests, an individual male was given the opportunity to copulate with three mature females under both hypoxic and normoxic conditions. In all tests of mating behaviour of male mosquito fish, the test environment referred to both the partial pressure of oxygen and the state of the female in the specific environment. Thus, when the mating behaviour of males was tested in hypoxia, the test environment refers to both low partial pressures of oxygen and exposure to females acclimated to this hypoxic environment. Differences between the acclimation groups in each specific environment were still due to underlying physiological and behavioural changes that were associated with acclimation. The observation tank consisted of a 30 cm × 30 cm × 20 cm deep glass aquarium with aged tapwater (pH 7.0), 2 cm gravel base, a corner aeration stone and a 200 W Jager heater. Black plastic was placed around the sides and back of the tank to reduce any external stimuli to the fish. An aquarium light was also placed above the tank for adequate illumination and to reduce the ability of the fish to detect movement in the darkened observation room. Only females that had not given birth during the previous 5 days were used in the observational experiments because of the possible effects of post-partum females on male behaviour (Farr, 1989). All females had a total body length of between 3.7–3.9 cm. In hypoxic conditions, only females acclimated to hypoxia were used for mating observations, and vice versa under normoxia.

Mating behaviour of each male was recorded for a total of 10 min and the observation time was started when the males first began to follow one of the females. Mating behaviours were entered into the Behavioural software program ETHOM 1.0 using a laptop computer. Total time each male spent following the females (TFT), the total number of mating attempts not including successful copulations and successful copulations were recorded for each individual during the observations. Mating attempts were defined as when a male swims towards a female, swings the gonopodium forward through an angle greater than 90°, and attempts to insert the intromittent organ into the female’s genital opening. Total number of mating attempts included both unsuccessful mating attempts and successful copulations. Copulations were deemed to occur when the gonopodium was inserted into the female’s genital opening. Copulations were easily distinguished from unsuccessful mating attempts by the characteristic twisting motion performed by the male following successful insertion (Wilson, 2005). This twisting away from the females is associated with the rapid removal of the barbed gonopodium from the female’s genital opening.

Male mating performance was then tested in a male–male competitive environment. Competitive experiments involved competing one male from each acclimation group against each other for the opportunity to copulate with a single female under both hypoxic and normoxic conditions. As body length is an important determinant of male territorial and sneaky-mating success in this species (McPeek, 1992; Pilastro et al., 1997), males from each acclimation group were first size-matched with an individual from the alternate acclimation group. Thus, 15 size-matched pairs of males were identified and tested. Each pair of size-matched males differed in body length by less than 0.1 mm. A small portion of the dorsal fin was removed from half of the males from each treatment so they could be distinguished in the observation tanks.

Mating behaviour of each competing male was assessed during a 20 min observation period in a 30 cm × 30 cm × 20 cm deep aquarium containing 1 mature female mosquito fish. The observation tank was identical to the set-up for the non-competitive tests. For each pair of fish, a different female was used for each test environment. To ensure differences in size between the males and females of each pair were kept constant across the test environment, each set of females were also size matched. Before observations, females were introduced into the observation tank and allowed to settle for at least 20 min. Males were then introduced into the aquarium and the observation period was started as soon as one male first began to follow the female.

During the 20 min observation period, several male mating behaviours were recorded and entered into the behavioural software program ETHOM 1.0 using a laptop computer. TFT, the total number of mating attempts and copulations were recorded for each individual during the competitive bouts. The total numbers of dominant behaviours displayed by each male towards the competing male (e.g. chasing male away, biting male) were also counted for each individual.

Total sperm number per stripped ejaculate was assessed for 17 hypoxic- and 18 normoxic-acclimated males. Ejaculates were stripped from each individual by palpating their abdomen under a dissecting microscope and diluting the ejaculate in 2 ml of 0.9% NaCl. A drop of this diluted medium was then placed on a sperm counting chamber (Hawksley Technology, Lancing, Sussex, UK; depth 10 μm, 0.1×0.1 calibration grid) and the total sperm number was counted within a known volume of solution (repeated three times with each sample). Males were separated from females for at least 24 h prior to removal of ejaculates to ensure sperm stores were unaffected by previous sexual behaviour (Matthews et al., 1997).

Statistical analyses

Data were analysed using the statistical programs R and SigmaStat. A two-way repeated-measures ANOVA was used to test for acute and acclimation effects on TFT. Total mating attempts, number of copulations and aggressive behaviours were analysed using R with a generalized linear mixed model (GLM) using penalized quasi-likelihood using a Poisson distribution to satisfy count data distributions. TFT was log_{10} transformed to satisfy normality assumptions. All data are presented as means ± s.e.m.

For male–male competitive interactions, TFT and ASR were analysed using one-way ANOVA with pair as a random factor in the statistical program R. Attempts, copulations and aggressive behaviours were analysed using a one-way ANOVA with pair as a random factor and a Poisson distribution for
Results

We found the effect of partial pressure of oxygen on $U_{max}$ differed significantly between treatment groups (two-way repeated measures ANOVA, $F_{1,60}=4.23$, $P=0.048$; Fig. 1). Although there was no difference between acclimation treatments when tested under normoxic conditions, the hypoxic-acclimated group had a $U_{max}$ that was significantly greater under hypoxic conditions than normoxic fish (post-hoc test; $P<0.05$).

Male mating behaviour in a non-competitive environment was influenced by long-term exposure to hypoxic conditions (Fig. 2). The relationship between partial pressure of oxygen and TFT was significantly influenced by acclimation treatment (two-way repeated measures ANOVA, $F_{1,2}=5.608$, $P=0.026$). When tested under hypoxic conditions, hypoxia-acclimated males spent $225.6±18.6$ s ($N=15$) following females, which was significantly greater than for normoxic-males ($130.0±24.3$ s $N=15$; Fig. 2A). By contrast, there was no significant interaction between treatment group and test environment for the total number of mating attempts (GLM, $t=-1.035$, $N=26$, d.f.=24, $P=0.311$; Fig. 2B). No difference between test environments (GLM, $t=-0.18$, $N=26$, d.f.=24, $P=0.86$) were detected for number of mating attempts. However, hypoxic-acclimated males obtained significantly greater number of copulations than normoxic males (GLM, $t=-3.84$, $N=26$, d.f.=24, $P<0.001$). Although no differences were detected between groups when tested under normoxic conditions, hypoxia-acclimated males (2.8±0.7 copulations $N=15$) obtained significantly more copulations in hypoxic conditions than normoxic-acclimated males (1.5±0.4 copulations $N=15$; Fig. 2C).

The mating behaviour of male mosquito fish during male–male competitive interactions was unaffected by long-term exposure to different levels of partial pressure of oxygen (Fig. 3). Although there was no significant interaction between test and acclimation environment on TFT (two-way repeated-measures ANOVA, $F_{1,60}=0.26$, $P=0.62$), both test (two-way repeated measures ANOVA, $F_{1,60}=12.15$, $P<0.005$) and acclimation environment (two-way repeated-measures ANOVA, $F_{1,60}=4.79$, $P=0.046$) independently influenced TFT (Fig. 3A). For example, hypoxia-acclimated males followed females for 70.5±13.4 s ($N=15$) in hypoxia and 102.2±16.2 s in normoxia (Fig. 3A). Test environment also significantly influenced the total number of mating attempts (GLM, $t=3.78$, $N=30$, d.f.=43, $P<0.005$), but there was no effect of long-term acclimation on mating attempts (GLM, $t=-1.45$, $N=60$, d.f.=43, $P=0.16$). Normoxic-acclimated males made 3.6±1.0 ($N=15$) mating attempts in hypoxic conditions and 6.3±1.4 ($N=15$) attempts in normoxic conditions (Fig. 3B). Total number of successful copulations did not differ between test environments (GLM, $t=1.66$, $N=60$, d.f.=43, $P=0.10$) or acclimation condition (GLM, $t=0.93$, $N=60$, d.f.=43, $P=0.36$; Fig. 3C). The total number of aggressive interactions between competing males was not influenced by either test environment (GLM, $t=1.54$, $N=60$, d.f.=43, $P=0.13$) or treatment group (GLM, $t=-1.44$, $N=60$, d.f.=43, $P=0.16$; Fig. 3).

During male–male competitive bouts, the relationship between test environment and aquatic surface respiration (ASR) was significantly influenced by acclimation treatment (two-way repeated measures ANOVA, $F_{1,60}=14.13$, $P=0.002$) and acclimation treatment (two-way repeated-measures ANOVA, $F_{1,60}=9.31$, $P=0.009$). When tested under hypoxic conditions, normoxic-acclimated males spent 153.5±40.6 s ($N=15$) in ASR, which was significantly greater than hypoxic-acclimated males (55.8±19.6 s $N=15$; Fig. 4; post-hoc test; $P<0.05$).

Male mosquito fish exposed to hypoxia for at least 6 weeks possessed $1.5×10^5±0.27×10^5$ ($N=17$) sperm cells per ejaculate, which was significantly fewer than the $3.9×10^5±0.83×10^5$ ($N=18$) for the normoxic males ($P<0.05$).

Discussion

The present study represents one of the first experimental investigations of the benefits of reversible phenotypic plasticity for reproductive performance. Previous tests of the benefits of acclimation responses have investigated the fitness consequences of developmental acclimation responses, with most studies rejecting the generality of the assumption that all acclimation changes are beneficial (Leroi et al., 1994; Zamudio et al., 1995; Huey and Berrigan, 1996; Bennett and Lenski, 1997; Huey et al., 1999; Wilson and Franklin, 2002). However, most physiological studies of acclimation deal with reversible responses in adult organisms, and the absence of information on the ecological significance of these responses represent an important gap in our understanding of the evolution of phenotypic plasticity. In this
study, we found that both locomotor performance and mating behaviour were substantially influenced by long-term exposure to low partial pressures of oxygen. Sustained swimming performance of male mosquito fish was greater in a hypoxic environment following long-term exposure to low partial pressures of oxygen. Similarly, in a non-competitive environment, male mosquito fish acclimated to hypoxic conditions spent a greater amount of time following females and obtained more copulations than normoxic-acclimated males when tested in low partial pressures of oxygen.

Improvements in the coercive mating ability of male mosquito fish with acclimation were most likely due to modifications in underlying physiological traits that were associated with aerobic capacity. Increased sustained swimming performance and decreased reliance on ASR indicate the greater aerobic capabilities of male mosquito fish in hypoxic conditions following acclimation to low partial pressures of oxygen. Previous studies reporting higher haematocrit, haemoglobin and erythrocyte concentrations of acclimated to hypoxic conditions (Frey et al., 1998; Timmerman and Chapman, 2004b; Jensen et al., 1993), suggest these traits may be the driving underlying physiological mechanisms. Timmerman and Chapman (Timmerman and Chapman, 2004b) also suggest that changes in lower critical oxygen tension and gill surface area in hypoxic-acclimated fish may support a greater aerobic capacity under hypoxic conditions.

Despite the advantages of a greater aerobic capacity for hypoxic-acclimated males in hypoxic environments, benefits to mating performance disappeared when tested in a competitive environment.

Fig. 2. Effect of partial pressure of oxygen on the mating behaviour (non-competitive environment) of male eastern mosquito fish Gambusia holbrooki acclimated to either hypoxic or normoxic conditions for at least 6 weeks. (A) The total time males spent following females, (B) total number of attempted copulations and (C) the total number of successful copulations recorded during a 10 min observation period in a non-competitive environment. Values are means ± s.e.m., N=15 for each acclimation group. *Significant results at P<0.05.

Fig. 3. Effect of partial pressure of oxygen on the mating behaviour (male–male competitive environment) of male eastern mosquito fish Gambusia holbrooki acclimated to either hypoxic or normoxic conditions for at least 6 weeks. (A) The total time males spent following females, (B) total number of attempted copulations and (C) the total number of successful copulations recorded during a 10 min observation period in a male–male competitive environment. Values are means ± s.e.m., N=15 for each acclimation group. *Significant results at P<0.05.
environment. We found no influence of long-term exposure to different partial pressures of oxygen on the mating behaviour of male mosquito fish in a competitive environment. Thus, it seems the mechanisms used to compensate for acute hypoxia, such as increasing opercular beat rate (Randall, 1970) and ASR (Kramer and Mehegan, 1981), seem to be able to procure sufficient oxygen to maintain reproductive behaviours in hypoxia for a short amount of time, before an oxygen debt is acquired (Vandenthillart and Verbeek, 1991). Given the benefits for the mating behaviour of male mosquito fish appear to be context dependent, interpreting the ecological significance of these reversible acclimation responses is difficult (Woods and Harrison, 2002). However, when tested in a hypoxic environment, the normoxic-males spent a greater proportion of time in ASR than hypoxic-acclimated males. Obtaining the same number of copulations whilst spending more time in ASR, may reflect a greater energetic cost for maintaining activity for the normoxic-acclimated males. Longer term analyses of mating behaviour and performance, lasting several hours, may better reflect the limitations of aerobic capacity on mating performance.

Total number of spermatozoa per stripped ejaculate was lower in males that were exposed to low partial pressures of oxygen. Long-term exposure to hypoxic conditions could be associated with increased energetic demands, such as increased costs of maintaining higher haemoglobin, myoglobin and gill filament surface area, that prevent the production of large quantities of sperm cells. In addition, prolonged hypoxia may lead to changes in hormone levels that can retard gonadal development. Hypoxia can produce endocrine disruption in fish (Wu et al., 2003; Perry et al., 2004; Shang and Wu, 2004). Testosterone, oestrogen and triiodothyronine were reported to decrease in common carp Cyprinus carpio when acclimated to hypoxia, resulting in retarded gonadal development (Wu et al., 2003). Increases in stress hormones such as cortisol as a result of chronic exposure to hypoxia (Pichavant et al., 2002) may disrupt spermatogenesis and other hormone pathways involved in sperm production. Alternatively, it is also possible that lower sperm numbers could be a physiological response to hypoxic conditions. Females may not be as reproductively active in these conditions, stimulating a down-production of sperm in males (Olsen and Liley, 1993).

Differences in copulation frequency between acclimation groups in the competitive and non-competitive environments may alternatively be related to the number of sperm available per ejaculate. In the non-competitive environment, hypoxia-acclimated males may have obtained more copulations because they possessed fewer sperm per ejaculate than the normoxia-acclimated males that may have allocated their sperm more prudently (Pound and Gage, 2004). By contrast, in a competitive environment, the lack of difference between the acclimation groups may be due to a need for all males to maximize copulation frequency, as predicted by sperm competition theory (Evans and Magurran, 2001). However, guppies (P. reticulata) with greater sperm reserves obtained a greater number of sneaky copulations than males with fewer sperm (Matthews et al., 1997), suggesting that normoxia acclimated males should attempt to maximize copulation frequency across all environments. Also, internally fertilizing poeciliids show mixed paternity broods and fertilization success is skewed towards the last male to copulate (Evans and Magurran, 2001). Thus, sperm number appears to be a poorer predictor of fertilisation success than the order of copulation (Evans and Magurran, 2001). Given this evidence, and the relentless reproductive behaviour of male mosquito fish, we suggest differences in sperm number and mating success between the acclimation groups must be a result of physiological performance in the environment and not male motivation.

Reversible physiological plasticity should be favoured when reliable cues indicate the state of the environment, and a fitness trade-off occurs between phenotypes expressed in each of the environments (Levins, 1968; Moran, 1992). Despite substantial improvements in locomotor and mating performance for the hypoxic-acclimated individuals in a hypoxic environment, there was no obvious trade-off in a normoxic environment. Some phenotypic modifications to hypoxic environments may even show short-term benefits in normoxia, supporting fitness-related aerobic behaviours. In the absence of a fitness trade-off, theory predicts that the phenotype should be constitutive rather than inducible (Moran, 1992). However, long-term costs of these modifications may outweigh short-term benefits. Long-term exposure to high partial pressures of oxygen was found to cause acid–base disturbances and an increase in the pH of blood in sea bass Dicentrarchus labrax (Cecchini and Caputo, 2003). A similar effect could occur in long-term exposure to high oxygen in mosquito fish acclimated to hypoxia, negating any benefits in short-term oxygen procurement. The reduction in sperm production in the hypoxic-acclimated individuals indicates this may be the case, and studies of the energetic costs of supporting a modified phenotype in the hypoxic environment may reveal further costs.

We found the benefits of reversible acclimation responses to

![Fig. 4. Effect of partial pressure of oxygen on the time (s) spent in aquatic surface respiration (ASR; male–male competitive environment) by male eastern mosquito fish Gambusia holbrooki acclimated to either hypoxic or normoxic conditions for at least 6 weeks. Values are means ± s.e.m., N=15 for each acclimation group. *Significant results at P<0.05.](image-url)
the mating performance of male mosquito fish were dependent on whether they were tested with or without male–male competition. In hypoxic environments, sustained aerobic activity and mating performance in the absence of male–male competition were substantially improved by acclimation to hypoxia. However, mating performance in a competitive environment was unaffected by long-term exposure to low partial pressures of oxygen. This represents one of the first experimental tests of the benefits of reversible acclimation responses, and suggests the ecological significance of physiological plasticity may be more complicated than previously thought.

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