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The role of the autonomic nervous system in control of cardiac and air-breathing responses to sustained aerobic exercise in the African sharptooth catfish *Clarias gariepinus*

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ABSTRACT

Clarias gariepinus is a facultative air-breathing catfish that exhibits changes in heart rate (f_H) associated with air-breaths (AB). A transient bradycardia prior to the AB is followed by sustained tachycardia during breath-hold. This study evaluated air-breathing and cardiac responses to sustained aerobic exercise in juveniles (total length ~ 20 cm), and how exercise influenced variations in f_H associated with AB. In particular, it investigated the role of adrenergic and cholinergic control in cardiac responses, and effects of pharmacological abolition of this control on air-breathing responses. Sustained exercise at 15, 30 and 45 cm s^{-1} in a swim tunnel caused significant increases in f_{AB} and f_H, from approximately 5 breaths h^{-1} and 60 heartbeats min^{-1} at the lowest speed, to over 60 breaths h^{-1} and 100 beats min^{-1} at the highest, respectively. There was a progressive decline in the degree of variation in f_H, around each AB, as f_{AB} increased with exercise intensity. Total autonomic blockade abolished all variation in f_H during exercise, and around each AB, but f_{AB} responses were the same as in untreated animals. Cardiac responses were exclusively due to modulation of inhibitory cholinergic tone, which varied from >100% at the lowest speed to <10% at the highest. Cholinergic blockade had no effect on f_{AB} compared to untreated fish. Excitatory β-adrenergic tone was approximately 20% and did not vary with swimming speed, but its blockade increased f_{AB} at all speeds, compared to untreated animals. This reveals complex effects of autonomic control on air-breathing during exercise in *C. gariepinus*, which deserve further investigation.

1. Introduction

The African sharptooth catfish *Clarias gariepinus* has bimodal respiration, possessing both well-developed gills and an air-breathing organ (Graham, 1997). The air breathing organ (ABO) comprises a pair of supra-branchial chambers, which contain extensive highly vascularized evaginations of the 2° and 4° gill arch known as the arborescent organs. The catfish rises to the surface and gulps air, which is then stored in the chambers from which oxygen diffuses into the bloodstream. *C. gariepinus* is a facultative air breathing fish; under routine normoxic conditions gill ventilation can meet the oxygen requirements of aerobic metabolism (Belão et al., 2011; McKenzie et al., 2015). This classification is based on experiments performed under routine conditions, it is not clear whether *C. gariepinus* might need to breathe air to meet increased oxygen demands of activities, in particular aerobic swimming (Graham, 1997, 2006; Lefevre et al., 2014). In all air breathing fishes studied to date, air breathing frequency (f_{AB}) increases during swimming activity (reviewed in Lefevre et al., 2014, 2016).

Some teleost fishes possess double cardiac innervation, so changes in heart rate (f_H) can be achieved by modulation of both excitatory adrenergic control, via β-adrenergic receptors, and inhibitory cholinergic control, via muscarinic receptors (Taylor et al., 1999; Sandblom et al., 2010). This modulation is used to ensure adequate oxygen and nutrient supply to all tissues, in response to changes in metabolic demand, such as digestion or sustained exercise. Unimodal water-breathing fishes show an increase in f_H and cardiac output with increased exercise (Randall, 1982; Chatelier et al., 2005; Olson and Farrell, 2006; McKenzie, 2011). This can be under both adrenergic and cholinergic control, and there is also the possibility of contributions from non-adrenergic and non-cholinergic (NANC) factors (Axelsson et al., 1989; Iversen et al., 2010). Cardiac responses to exercise, and their autonomic control, have not been described in any air-breathing fish species.

This is of some interest because all air-breathing fishes studied, to date, exhibit profound variations in cardiac activity associated with each air-breathing event (AB) (Johansen, 1966; Graham, 1997; Belão et al., 2011, 2015). As the fish surfaces to expire the gas from its ABO,

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it exhibits a transient bradycardia and then, after it has inspired fresh air, it exhibits a more sustained tachycardia. These variations in f_H around each AB event appear largely to be under inhibitory cholinergic control (Graham et al., 1995; McKenzie et al., 2007a; Iversen et al., 2011; Teixeira et al., 2015). It has been suggested that they optimize O_2 uptake by the ABO (Johansen, 1966; Graham et al., 1995; Skals et al., 2006). This remains, however, to be proven, as pharmacological blockade of all cardiac autonomic control did not affect f_{AB} and rates of aerial oxygen uptake by the jeju *Hoplerythrinus unitaeniatus* in profound aquatic hypoxia (McKenzie et al., 2007a). Aerobic exercise may represent a means of investigating the functional significance of f_H modulation during air-breaths in bimodal fishes, as increased oxygen demands of exercise may require more effective oxygen uptake from the ABO.

Thus, the aim of this study was to evaluate the air-breathing and cardiac responses of *C. gariepinus* to sustained aerobic exercise. In particular, to investigate the role of adrenergic and cholinergic control in cardiac responses, and the effects of pharmacological abolition of this control on air-breathing responses. The hypotheses were that (1) f_{AB} would increase with the intensity of exercise; (2) f_H would also increase with exercise and all related changes in f_H would be largely under inhibitory cholinergic control, and (3) pharmacological blockade of cardiac autonomic control would compromise the efficiency of air-breathing, leading to higher f_{AB} at any given level of aerobic exercise.

2. Materials and methods

2.1. Experimental animals

Juvenile *C. gariepinus* of unknown sex, with a mass of ~130 g and total length of ~20 cm were obtained from Piscicultura Polettini (www.facebook.com/cesar.polettini) and transported to the Federal University of São Carlos (UFSCar), São Carlos (SP), Brazil. They were maintained in indoor tanks of 1000 l provided with a recirculating flow of biofiltered freshwater at 25 °C, under a natural photoperiod, for at least 1 month prior to experiments. They were fed ad libitum each day with commercial pellets (22% protein diet), but individuals were fasted for at least 48 h prior to the trials.

2.2. Animal preparation

Fish were anesthetized in a benzocaine solution (0.1 g l^{-1}); predissolved in 3 ml of 70% ethanol. Once active gill ventilation had ceased, fish were placed ventral side up on a surgical table and their gills artificially ventilated with an aerated benzocaine solution (0.05 g l^{-1}). Fishes were fitted with electrocardiogram (ECG) electrodes as described previously (Belão et al., 2011). Briefly, one electrode (+) was inserted under the skin in a ventral position below the orobranchial cavity anterior to the heart, the second (−) inserted ventrally close to the pectoral fins, posterior to the heart. The electrodes were held in place with sutures. In addition, a polyethylene cannula (PE10) filled with a solution of 0.9% NaCl was inserted into the peritoneal cavity via a small incision, advanced towards the heart, and then fixed with sutures on the body wall, as described previously (McKenzie et al., 2007a; Iversen et al., 2010; Teixeira et al., 2015). The cannula and ECG wires were then brought round to the front of the dorsal fin, where they were held in place with a common suture, such that they trailed above the fish during swimming protocols. After surgery each fish was transferred to the swimming respirometer and allowed at least 24 h recovery in aerated water at a current flow of 10 cm s^{-1} (i.e. below half a body length s^{-1}) at the experimental temperature of 25 °C.

2.3. Aerobic exercise protocols

Fish were exercised in a Steffensen-type swim-tunnel respirometer constructed of Plexiglas®, designed and built in the School of Biosciences, University of Birmingham, to exercise fish in a non-turbulent water flow with a uniform velocity profile (for details see McKenzie et al., 2007b). The swim tunnel was modified to allow fish to breathe air during exercise, as described in McKenzie et al. (2012). The anterior portion of the swim tunnel was shielded with black plastic sheeting to avoid visual disturbance to the catfish, which could, however, be observed from behind. Fish rested on the bottom with occasional gentle swimming movements, in particular when they rose up through the water column to take an air breath (AB).

On the morning of the swim trial, the current speed was raised to 15 cm s^{-1}, which elicited gentle sustained swimming movements. The fish were maintained at this speed for 60 min, during which time they all took at least two air breaths (ab). The swimming speed was then increased to 30 cm s^{-1} for 15 min. At this speed, the catfish swam steadily and actively, with much more frequent AB. Swimming speed was then raised to 45 cm s^{-1} for 15 min, at which speed the catfish swam and breathed air vigorously. The swimming speeds were chosen to elicit sustained aerobic swimming, avoiding any potential anaerobic bursting activities and possibility of fatigue. Pilot experiments found that faster speeds (60 cm s^{-1}) did cause bursting activities and could not be sustained, indicating that maximum aerobic swimming speed for this species, at the prevailing bodylength, lies somewhere between 2 and 3 bodylengths s^{-1}. The speed was then returned to 15 cm s^{-1} for 90 min, to ensure that the fish had returned to a steady state. Pilot experiments demonstrated that this interval was more than adequate to ensure that air-breathing and heart rate returned to frequencies observed at 15 cm s^{-1} prior to the test.

After this control trial, catfish were injected with drugs to elicit autonomic blockade, and then exposed to the same swimming protocol at 15, 30 and 45 cm s^{-1}. Two series of drug injections were performed, each with a different sequence of β-adrenergic and cholinergic blockade, to evaluate possible interactions between adrenergic and cholinergic tones (Altimiras et al., 1997). In group one ($n = 9$) the fish were injected with 5 mg kg^{-1} propranolol hydrochloride (dissolved in 0.5 ml saline) to cause β-adrenergic blockade, with 30 min allowed for the drug to exert its effects. They were then maintained at 15 cm s^{-1} for 60 min, during which time they all took at least two AB. Speed was then increased to 30 and subsequently to 45 cm s^{-1}, as described above. They were then returned to 15 cm s^{-1} for 90 min, to recover from exercise. At that point they were injected with a cocktail of 4 mg kg^{-1} atropine sulfate and 2 mg kg^{-1} propranolol hydrochloride (dissolved in 0.5 ml saline) to cause total autonomic blockade. After 30 min for the drugs to exert their effects, they were given the same swim protocol as described above. Group two ($n = 9$) were treated exactly as for Group 1 but, following the control swim, were injected with 4 mg kg^{-1} atropine sulfate to elicit cholinergic muscarinic blockade, followed by injection of 5 mg kg^{-1} propranolol hydrochloride and 2 mg kg^{-1} atropine sulfate to attain double blockade. Drug doses were based upon previous studies on fishes (reviewed in Altimiras et al., 1997), to elicit complete receptor saturation. All chemicals were obtained from Sigma- Aldrich (www.sigmaaldrich.com).

2.4. Air breathing and heart rate frequencies

Air breathing frequency (f_{AB}) was observed visually and then calculated in breaths h^{-1} based on the mean interval between AB. To record heart rate (f_H), ECG electrodes were connected to an amplifier (Animal Bio Amp-ADInstruments, www.adinstruments.com) of a data acquisition system (Power lab-ADInstruments). This system was connected to a computer and ECG was displayed and recorded throughout the protocol with ADInstruments Lab Chart 7 software. Care was taken to place an event marker on the ECG trace at the moment that the fish broke the surface to take an AB.

The routine f_H was calculated for both groups at all swim speeds based on the time incurred for 10 R-R intervals at the midpoint between two AB, then converted to beats min^{-1}. Furthermore, AB events caused marked changes in f_H (Belão et al., 2011; Teixeira et al., 2015) and

instantaneous f_H was calculated for 10 beats before and after each AB (f_H Pre-AB and f_H Post-AB, respectively) for both groups at all swim speeds, based upon the relevant R-R intervals.

2.5. Calculation of adrenergic and cholinergic tones

The relative adrenergic and cholinergic tones were calculated based on R-R interval ($1/f_H$) as described by Altimiras et al., (1997).

When propranolol was injected before atropine:

$$\text{Adr}(\%) = \frac{(R-R)_\beta - (R-R)_{cont}}{(R-R)_0} * 100$$

$$\text{Chol}\ (\%) = \frac{(R-R)_\beta - (R-R)_0}{(R-R)_0} * 100$$

When atropine was injected before propranolol:

$$\text{Chol}(\%) = \frac{(R-R)_{cont} - (R-R)_{musc}}{(R-R)_0} * 100$$

$$\text{Adr}(\%) = \frac{(R-R)_0 - (R-R)_{musc}}{(R-R)_0} * 100$$

where $(R-R)_{cont}$ is the pulse interval of untreated fish and $(R-R)_0$ is the pulse interval after complete autonomic blockade. $(R-R)_\beta$ and $(R-R)_{musc}$ are the pulse intervals after β-adrenoceptor and cholinergic muscarinic blockade, respectively. Chol(%) and Adr(%) are the cholinergic and adrenergic tones, in percent.

2.6. Supporting pharmacological studies

To ensure that the doses of atropine and propranolol were effectively blocking cholinergic and adrenergic control of the heart in *C. gariepinus*, acetylcholine chloride (1 ml kg^{-1} of 10^{-4} mol l^{-1}) and then adrenaline (1 ml kg^{-1} of 10^{-4} mol l^{-1} solution) were infused via the cannulae 1 h after infusion of the respective antagonists and resultant changes in ECG were recorded. Neither of the agonists elicited any alterations in f_H, confirming previous results using this same surgical and pharmacological protocol (McKenzie et al., 2007a).

2.7. Statistical analyses

Data were tested for normality using Shapiro-Wilk test prior to parametric tests. Within each group, the influence of treatment on different variables (f_{AB}, f_H, % action of tonus) was analyzed using two-way ANOVA for repeated measures with one factor being swim test (i.e. type of pharmacological blockade) and the repeated factor being swim speed. When a significant effect was observed, Holm-Sidak post-hoc tests were used to identify where differences among means occurred. Effects were considered statistically significant at $P \leq 0.05$. Results are presented as means ± SEM. All analyses were performed with Sigma Plot 13.0 (Systat Software Inc., www.systat.org).

3. Results

3.1. Effects of sustained exercise and pharmacological blockade on air breathing

Sustained exercise elicited a significant increase in mean f_{AB} with swimming speed in *C. gariepinus*, within both groups for all swim tests (Table 1). After each swim test f_{AB} dropped very rapidly and remained low during the 90 min recovery period (data not shown). Mean f_{AB} at 15 cm s^{-1} was similar in the three sequential swim tests in both groups (Table 1).

In group 1, for the control test there was a significant increase in mean f_{AB} from 15 to 30 cm s^{-1} but no further increase from 30 to 45 cm s^{-1}. Following injection of propranolol, mean f_{AB} also increased as the fish increased speed from 15 to 30 and then 45 cm s^{-1}, and was significantly higher than the control values at 30 and 45 cm s^{-1} (Table 1). Following double blockade, mean f_{AB} once again increased as swim speed rose from 15 to 30 and then 45 cm s^{-1}. At 30 cm s^{-1}, mean f_{AB} of the double blocked fish was not different from either control or propranolol values but, at 45 cm s^{-1}, it was higher than for the control swim (Table 1). In group 2, mean f_{AB} increased progressively in all three sequential swim tests, as speed rose from 15 to 30 to 45 cm s^{-1}. Injection of atropine and then double blockade did not elicit any significant differences in mean f_{AB} compared to the control swim test (Table 1). After double blockade, however, mean f_{AB} at 45 cm s^{-1} was lower than as measured after atropine alone (Table 1).

3.2. Effects of sustained exercise and pharmacological blockade on routine f_H, and associated autonomic tones

Within both groups, the control swim test was associated with a significant increase in mean f_H across all three speeds (Table 2). Furthermore, as soon as current speed was returned to 15 cm s^{-1} to recover from the control swim, f_H dropped very rapidly prior to the first drug injection (data not shown).

In group 1, injection of propranolol had no significant effect on mean f_H compared to the control swim test, at any speed, and there was still an incremental increase in f_H across the three sequential speeds. After double blockade mean f_H was very high and invariant across the three swim speeds (Table 2). In group 2, injection of atropine caused mean f_H to increase very significantly compared to the control swim test, and it was then invariant across all speeds. Subsequent double blockade caused mean f_H to decline significantly compared to atropine alone, but it was still significantly higher than the control test and remained invariant across all speeds (Table 2).

Adrenergic tone was around 20% at all swim speeds, with no significant effect of swimming speed although it decreased to a mean value of about 10% at 45 cm s^{-1} in group 1 fish (Fig. 1). Cholinergic tone was high at the lowest swim speed and then declined progressively as swim speed increased (Fig. 1), indicating that the progressive increase in mean routine f_H with swim speed in the control swim tests (Table 2) was due to a progressive removal of inhibitory cholinergic tone. Mean cholinergic tone was noticeably higher in Group 1 compared to Group 2, note that the two groups were studied sequentially.

Table 1
Air breathing frequency (f_{AB}: breaths·h^{-1}) of *Clarias gariepinus* during exercise at three sustained speeds of swimming (15, 30 and 45 cm s^{-1}).

		Swimming speed (cm s^{-1})		
		15	30	45
Group I	Control	4,9 ± 0,9[a]	31,6 ± 3,8[b]	34,1 ± 3,3[b]
	Propranolol	7,4 ± 1,5[a]	51,8 ± 7,2[b*]	68,4 ± 7,9[c*]
	Double Blockade	5,7 ± 2,1[a]	43,1 ± 22,2[b]	65,5 ± 6,0[c*]
Group II	Control	4,5 ± 0,8[a]	35,9 ± 6,3[b]	67,6 ± 8,0[c]
	Atropine	4,1 ± 1,2[a]	29,6 ± 3,5[b]	75,6 ± 11,6[c†]
	Double Blockade	5,7 ± 0,8[a]	39,1 ± 6,0[b]	54,9 ± 4,2[c]

Values are presented as mean ± SEM; $n = 9$ ($P \leq 0,05$). Different letters indicates significant difference between the swimming speeds at each protocol. (*) indicates significant difference in relation to Control within each group at the same swimming speed. (†) indicates significant difference in relation to Double Blockade to within each group at the same swimming speed.

Table 2
Routine heart rate (Routine f_H: bpm) of *Clarias gariepinus* during exercise at three sustained speeds of swimming (15, 30 and 45 cm s^{-1}).

		Swimming speed (cm s^{-1})		
		15	30	45
Group I	Control	53 ± 3^{a}	72 ± 4^{b}	87 ± 6^{c}
	Propranolol	$49 \pm 4^{a\dagger}$	$67 \pm 4^{b\dagger}$	$80 \pm 3^{c\dagger}$
	Double Blockade	$95 \pm 5^{a*}$	$99 \pm 5^{a*}$	$102 \pm 5^{a*}$
Group II	Control	66 ± 2^{a}	93 ± 4^{b}	113 ± 4^{c}
	Atropine	$122 \pm 4^{a*\dagger}$	$122 \pm 3^{a*\dagger}$	$121 \pm 3^{a\dagger}$
	Double Blockade	$100 \pm 5^{a*}$	$101 \pm 7^{a*}$	$102 \pm 7^{a*}$

Values are presented as mean ± SEM; $n = 9$ ($P \leq 0{,}05$). Different letters indicate significant differences between the swimming speed at each protocol. (*) indicates significant difference in relation to Control within each group at the same swimming speed. (†) indicates significant difference in relation to Double Blockade within each group at the same swimming speed.

3.3. Effects of sustained exercise and pharmacological blockade on f_H pre- and post-AB, and associated autonomic tones

In both groups, for the control swim (Fig. 2A and D), instantaneous f_H was stable for 10 beats before the breath, based on R-R interval. A transient bradycardia occurred over a single beat at the breath, followed by a clear tachycardia that remained relatively stable over the ensuing 10 beats (Fig. 2A and D). The increase in f_H with swim speed was evident and appeared to be more marked for f_H Pre-AB than for f_H Post-AB (Fig. 2A and D).

Injection of propranolol caused a slight decline in f_H compared to the relevant control swim test (Fig. 2B) but the catfish continued to show the transient bradycardia for a single beat as they breathed air, followed by a sustained tachycardia (Fig. 2B). Injection of atropine, whether alone or in double blockade, abolished all changes in f_H associated with AB events (Fig. 2C, E, F).

The calculations of autonomic tones revealed that, as observed for routine f_H, β-adrenergic tone was statistically invariant under all circumstances (Fig. 3). As observed for routine f_H during swimming, any changes in f_H around AB events (Fig. 3A, B, D) were a result of modulation of inhibitory cholinergic tone. That is, at 15 cm s^{-1}, inhibitory cholinergic tone declined between pre- and post-AB. The increase in swim speed to 30 and then 45 cm s^{-1} was associated with a sequential significant reduction in cholinergic tone before the AB, but not after the AB. At 30 cm s^{-1}, cholinergic tone still declined significantly between pre- and post-AB, but this effect was no longer significant at 45 cm s^{-1} (Fig. 3). Thus, cholinergic tone before the AB was progressively released as f_H increased with swim speed. After the AB, cholinergic tone was almost completely released. It was generally low and statistically similar across all speeds, with the mean value being extremely low at 45 cm s^{-1} in both groups, but especially in group 2 (Fig. 3).

4. Discussion

The results revealed that sustained aerobic exercise stimulated significant increases in air breathing and heart rate, and affected the changes in heart rate associated with air- breathing events, in *C. gariepinus*. They also revealed that all changes in heart rate associated with swimming and air-breathing were due to modulation of inhibitory cholinergic tone.

4.1. Air breathing responses to exercise and effects of autonomic blockade

The increase in f_{AB} with swimming speed presumably reflected a reflex response to meet the increased metabolic demands of exercise. Previous studies with bimodal fishes, such as *Amia calva*, *Lepisosteus oculatus*, *Megalops cyprinoides*, *Gymnotus carapo* and *Pangasianodon hypophthalmus*, reported the same pattern of response (Farmer and Jackson, 1998; Seymour et al., 2007; McKenzie et al., 2012; Lefevre et al., 2013, 2014) but this study is the first to investigate how such responses might be linked to autonomic control of the heart.

Mechanisms of stimulation and regulation of f_{AB} during exercise in normoxic conditions are not yet known (Lefevre et al., 2014). One possible mechanism is a limitation to O_2 extraction (EO_2) by the gills (Packard, 1974). Belão et al. (2011) reported an EO_2 of around 49% in *C. gariepinus* in normoxic resting conditions, which is low compared with some unimodal species, such as *Cyprinus carpio, Hoplias malabaricus* and *Oreochromis niloticus* at 90%, 83% and 80%, respectively (Lomholt and Johansen, 1979; Rantin et al., 1992; Fernandes and Rantin, 1994). This greater ability to extract O_2 in unimodal fishes is usually associated with a relatively larger branchial respiratory surface area (Fernandes et al., 1994). In many bimodal fishes the evolution of the ABO has been associated with a reduction in gill surface area (Graham, 1997), which would reduce gill EO_2. Even when compared with other bimodal species, like *Hoplerythrinus unitaeniatus*, *Synbranchus marmoratus* and *Ancistrus chagresi*, at 71%, 67% and 79%, respectively (Eduardo et al., 1979; Graham, 1983; Oliveira et al., 2004), the EO_2 of *C. gariepinus* is relatively low.

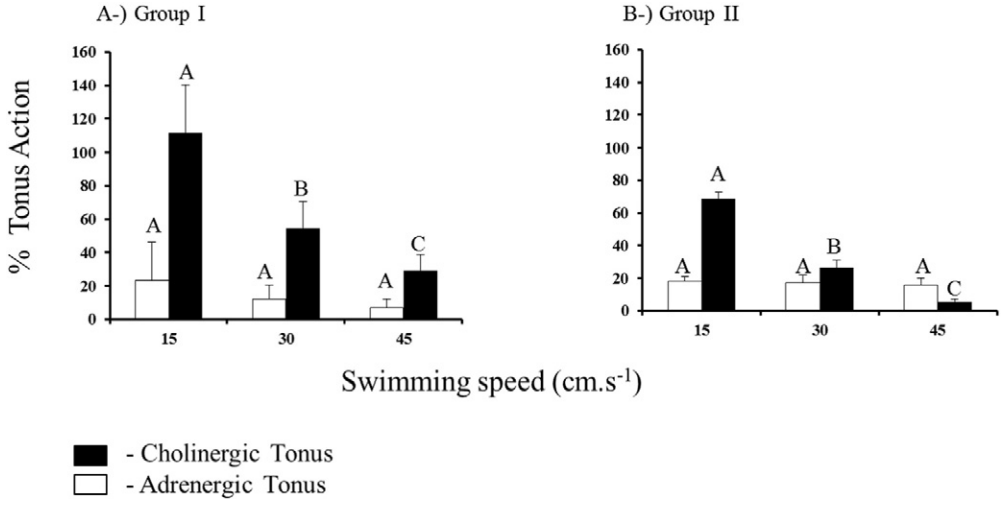

Fig. 1. Autonomic tone on the heart of *Clarias gariepinus* at three different sustained swimming speed (15, 30 and 45 cm s^{-1}). Black and white bars represent respectively, cholinergic and adrenergic tone action calculated from measured f_H at each swimming speed between air breaths. Different letters indicate significant differences between the proper tone at different swimming speeds. Values are mean ± SEM; $n = 9$ ($P \leq 0{,}05$).

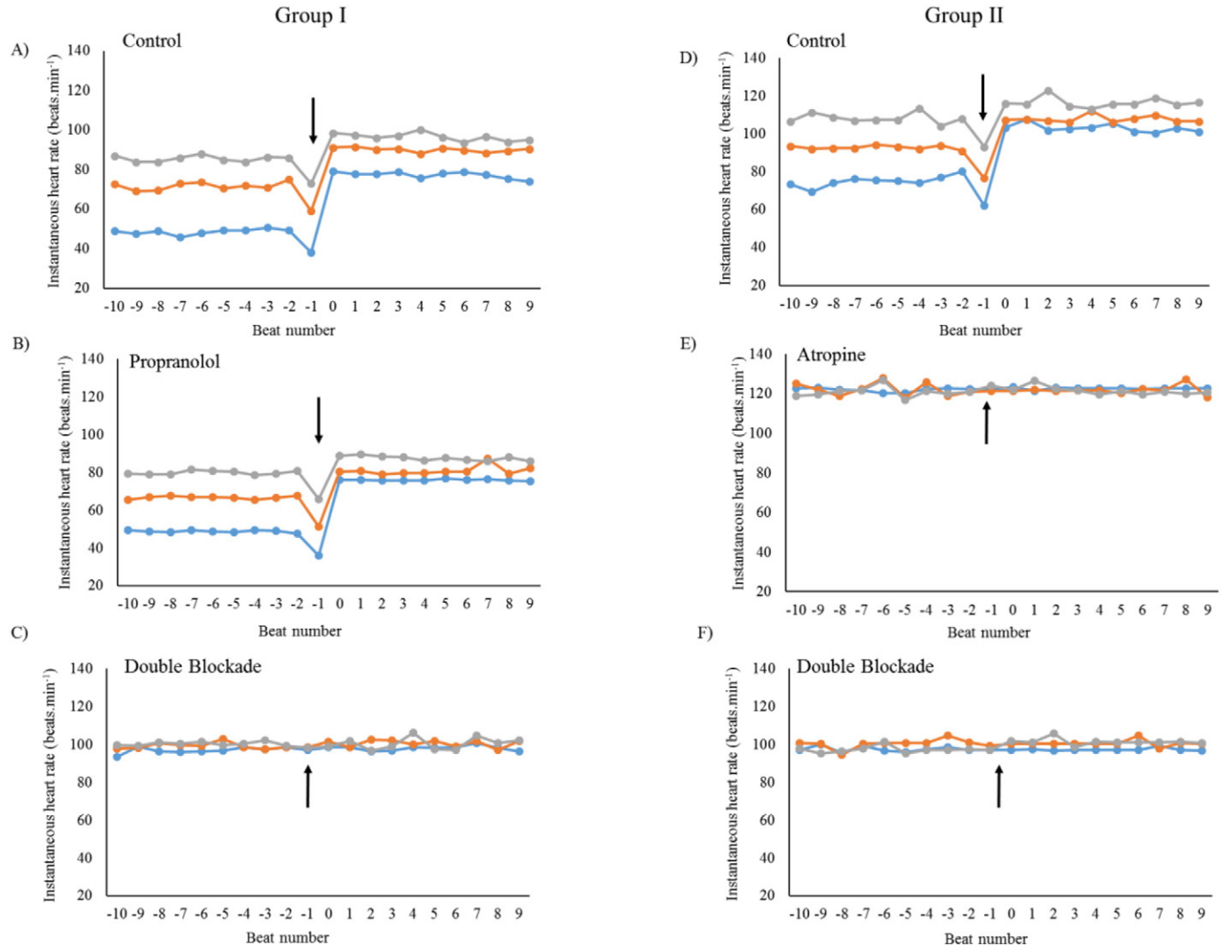

Fig. 2. Instantaneous heart rate (beats min^{-1}) in nine *Clarias gariepinus* in each experimental group, calculated from mean R-R interval over 10 heartbeats prior to and succeeding each air breath. The colours represent different swimming speeds: blue: 15 cm s^{-1}; red: 30 cm s^{-1}; purple: 45 cm s^{-1}. The black arrow indicates the visual record of an air breath.

Thus, if the gills of *C. gariepinus* have a limited capacity for EO_2, they may have to increase reliance on air-breathing to meet the increased metabolic demands of exercise. This argument, however, cannot explain why some air-breathing fishes increase air-breathing frequency during aerobic exercise even when they can, in fact, achieve the same aerobic scope and aerobic swimming performance using gill ventilation alone, if denied access to air (McKenzie et al., 2012; Lefevre et al., 2014, 2016). Another possible mechanism underlying the increased AB frequency during sustained swimming could be a decrease in O_2 supply to the heart, an essential organ for performance of aerobic exercise (Graham and Farrell, 1990; Farrell and Clutterham, 2003). A reduction in venous blood PO_2, due to increased extraction of oxygen by working skeletal muscle, may have stimulated O_2 chemoreceptors in the venous circulation, so stimulating f_{AB} (McKenzie et al., 2012; Lefevre et al., 2014). In *C. gariepinus* there is evidence that these chemoreceptors are located mainly on the first gill arch but may occur in other places, including the ABO, which is an evagination of the gills (Belão et al., 2015).

It is very interesting that β-adrenergic blockade with propranolol caused higher f_{AB} when the catfish were swimming at 30 and 45 cm s^{-1}. This finding may reflect effects of propranolol on gill perfusion; adrenaline is known to cause branchial vasodilation while acetylcholine elicits branchial vasoconstriction, increasing vascular resistance (Johansen et al., 1968). In rainbow trout *Oncorhynchus mykiss*, β-adrenergic gill receptors vasodilate lamellar afferent arterioles, decreasing vascular resistance, therefore increasing perfusion of the respiratory surfaces (Perry et al., 1985; Vermette and Perry, 1988), increasing functional surface area and, consequently, the uptake of O_2 (Sundin and Nilsson, 2002). Furthermore, propranolol can reduce rates of gill ventilation in some unimodal fishes (McKenzie et al., 1995) and, in the bimodal *H. unitaeniatus*, β-adrenergic blockade in profound hypoxia caused rates of oxygen uptake from water to fall to zero (McKenzie et al., 2007a). Thus, β-adrenergic blockade in the catfish may have compromised gill perfusion and ventilation, so reducing rates of aquatic oxygen uptake, and increasing reliance on aerial oxygen uptake, and f_{AB}, during exercise.

It is also interesting that double blockade abolished this effect of propranolol in Group 1, returning air-breathing responses to the pattern observed in untreated animals, and that cholinergic blockade alone, in Group 2, had no effects on air-breathing responses to exercise. That is, although the data indicate that air-breathing activity can be sensitive to β-adrenergic blockade, the mechanisms underlying the complex effects of the adrenergic versus cholinergic blockade require further investigation.

4.2. Cardiac responses and autonomic tones during exercise

The pharmacological blockades revealed a clear dominance of inhibitory vagal control on regulation of f_H during exercise. The cholinergic

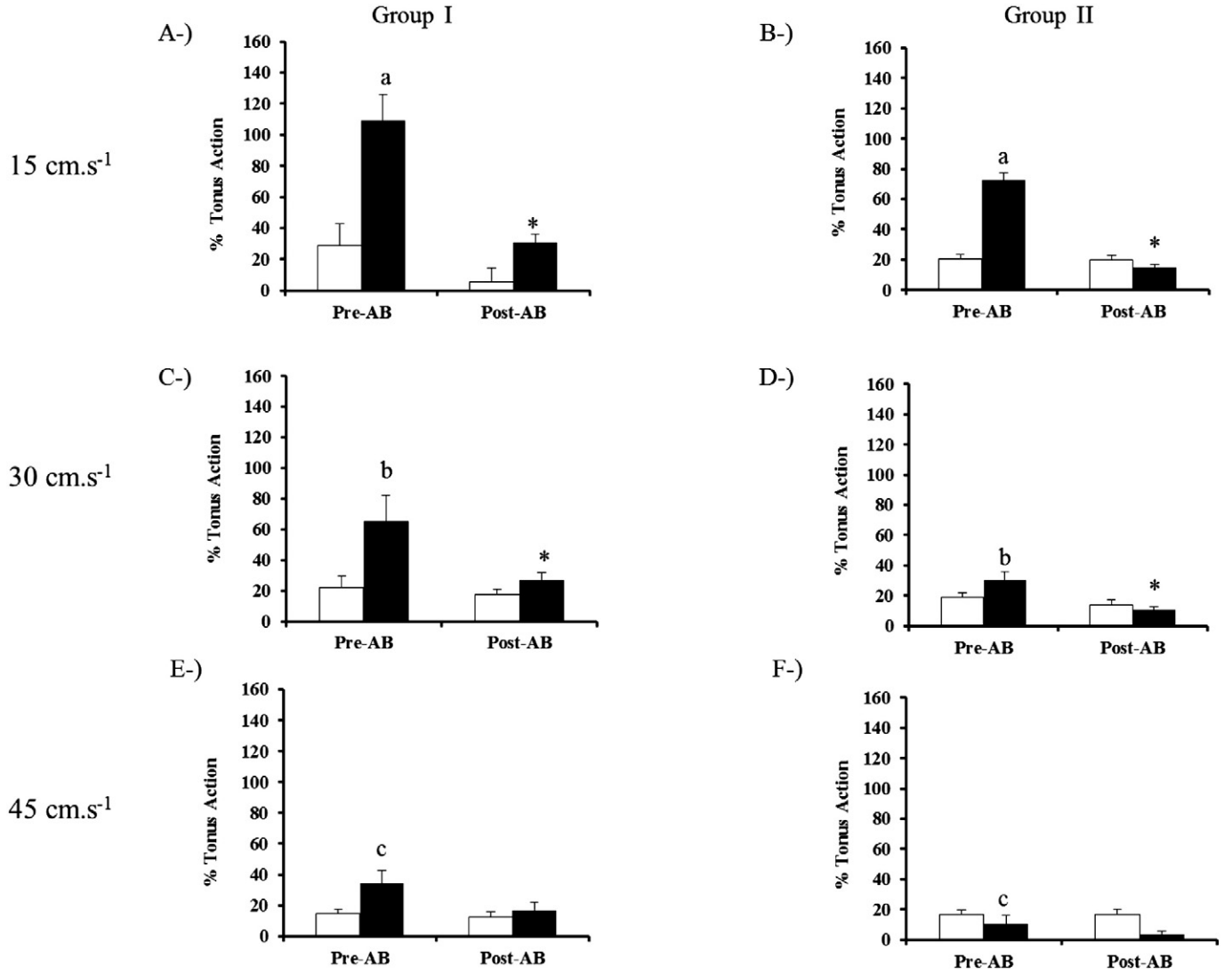

Fig. 3. Autonomic tone on the heart of *Clarias gariepinus* both before and air breath (Pre-AB) and after an air breath (Post-AB) at three different sustained swimming speed (15, 30 and 45 cm s^{-1}). Black and white bars represent respectively, cholinergic and adrenergic tone. Different letters indicate significant differences at Pre- or Post-conditions between the mean tones at different swimming speeds; (*) indicates significant differences between mean Pre- and Post-tones at the same swimming speed. Values are mean ± SEM; $n = 9$ ($P \leq 0{,}05$).

dominance over f_H under resting conditions, and the subsequent release of inhibition with increased swimming activity, have been reported in other fishes, like *Dicentrarchus labrax, Carassius auratus*, *Gadus morhua*, *Pagothenia bernacchi* and *Protopterus annectens* (Cameron, 1979; Axelsson and Nilsson, 1986; Axelsson et al., 1992; Iversen et al., 2010; Sandblom et al., 2010). A cholinergic dominance is not found in all species. Axelsson et al. (1989) showed a role of increased stimulatory adrenergic tone contributing to a rise in f_H during exercise in *Hemitripterus americanus*. Although adrenergic tone did not change as f_H increased during exercise in *C. gariepinus,* a clear adrenergic tone was revealed when propranolol was administered after atropine, with a constant excitatory tone of about 20% at all swimming speeds. The injection of propranolol and removal of acceleratory β-adrenergic tonus in Group 1 fish did not cause a significant bradycardia, which contrasts with findings from unimodal water breathing fishes (Cameron, 1979; McKenzie et al., 1995; Leite et al., 2009; Iversen et al., 2010). This may be due to an interaction of tones known as "compensatory demand" (Altimiras et al., 1997), whereby adrenergic blockade caused a compensatory release of inhibitory cholinergic tone, such that f_H did not change significantly compared to the control group. That is, having a large vagal tone apparently provides scope for *C. gariepinus* to modulate f_H irrespective of any parallel adrenergic tone. The data also demonstrate clearly that there was no regulation of f_H by NANC factors during exercise, because after double blockade it remained invariant during the swim protocol.

It is not clear why Group 2 had a markedly lower cholinergic tone than Group 1. The two groups were studied sequentially, were from two separate cohorts and were studied in two different laboratories within the same building. Any of these factors could have contributed to the observed effect, in particular the second group was studied in a laboratory with a greater degree of disturbance than the first. This result was unforeseen and, in hindsight, it would of course have been preferable to investigate the two groups simultaneously and on the same cohort. Although this difference in tone cannot be explained at present, it does not affect the general conclusions of the study, as the pattern of f_{AB} and f_H responses to exercise were similar in both groups, with a progressive increases in air-breathing and a tachycardia, and loss of the degree of variation in f_H around each AB event, with all changes in f_H being under inhibitory cholinergic control.

C. gariepinus showed a cardiac response around each AB, characterized by a pre-breath bradycardia represented as a single missed heartbeat and a more sustained post-breath tachycardia, similar to observations in all bimodal fishes studied to date (e.g. Belão et al., 2015; Farrell, 1978; Johansen, 1966; McKenzie et al., 2007b).

Cholinergic blockade raised heart rate and abolished the cardiac responses to surfacing in *C. gariepinus*, identifying parasympathetic control as the efferent arm of the response. It is only possible to speculate regarding the reflexogenic origins of this cardiac response, particularly as air breaths in the catfish were recorded visually so that the detailed temporal sequence of the surfacing event was not available. The pre-breath cardiac interruption could be a response to overall postural change or the orobranchial movements that anticipate taking a breath while the onset of the post-breath tachycardia could be due to mechano-receptor stimulation during inflation or filling of the ABO, followed by stimulation of chemoreceptors in the ABO and cardiovascular system (Taylor and Wang, 2009; Taylor et al., 2014). This response appears largely to be under inhibitory cholinergic control in bimodal fishes (Graham et al., 1995; McKenzie et al., 2007b; Iversen et al., 2011; Teixeira et al., 2015). The present results confirm a complete vagal control over the changes of f_H around each AB in *C. gariepinus*, decreasing its activity after the event, while adrenergic tone remained constant.

It has been proposed that these variations in f_H around each AB serve to optimize ABO perfusion after the breath (Johansen, 1966; Skals et al., 2006), and so optimize oxygen uptake from it, especially in situations of high energetic demand (Farmer and Jackson, 1998; Graham et al., 1995). In fact, in *C. gariepinus*, increasing exercise intensity was associated with a decline in the degree of variation in f_H around each AB. Furthermore, there was no evidence that abolition of all autonomic control of the heart compromised the efficiency of air-breathing during exercise, as f_{AB} after total pharmacological blockade was not systematically higher than f_{AB} under control conditions. Total blockade did, of course, cause f_H to be very high under all circumstances, which may have obscured any beneficial effects of increasing f_H after an AB. Further work is, therefore, required to understand the functional significance of the changes in f_H associated with air-breathing in bimodal fishes.

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