Journal of Zoology

Journal of Zoology. Print ISSN 0952-8369

Biochemical correlates of aggressive behavior in the Siamese fighting fish

M. D. Regan[1], R. S. Dhillon[1,2], D. P. L. Toews[1], B. Speers-Roesch[3], M. A. Sackville[1], S. Pinto[1], J. S. Bystriansky[4] & G. R. Scott[5]

1 Department of Zoology and Biodiversity Research Centre, University of British Columbia, Vancouver, British Columbia, Canada
2 Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI, USA
3 Department of Ocean Sciences, Memorial University of Newfoundland, St. John's, Newfoundland, Canada
4 Department of Biological Sciences, DePaul University, Chicago, IL, USA
5 Department of Biology, McMaster University, Hamilton, Ontario, Canada

Keywords
comparative physiology; *Betta splendens*; aggression; metabolism; energy supply.

Correspondence
Matthew D. Regan, Department of Zoology and Biodiversity Research Centre, University of British Columbia, 6270 University Blvd., Vancouver, British Columbia V6T 1Z4, Canada.
Email: regan@zoology.ubc.ca

Editor: Nigel Bennett

Received 12 November 2014;
revised 20 April 2015;
accepted 24 April 2015

doi:10.1111/jzo.12259

Abstract

The Siamese fighting fish *Betta splendens* is a facultative air-breathing freshwater fish often used for studying the physiological bases of agonistic behaviors. The agonistic interactions typical of paired male conspecifics are energetically costly, as indicated by associated increases in whole-animal metabolic rate. However, little is known about the biochemical responses that underlie metabolism and performance during agonistic encounters. We examined biochemical indices of aerobic and anaerobic energy metabolism in locomotory muscle to ascertain whether they are important correlates of aggressive behavior. Pairs of male *B. splendens* were allowed to interact aggressively across a glass partition while their behaviors were quantified (striking, opercular flaring, surfacing for air) and the levels of several key metabolites (including lactate, creatine phosphate, free creatine, adenosine triphosphate, free adenosine diphosphate) and activities of enzymes of aerobic (citrate synthase) and anaerobic (pyruvate kinase, lactate dehydrogenase) metabolism were assayed in their muscle at the end of the trial. More aggressive fish (those exhibiting a higher strike frequency) had higher surfacing frequencies (presumably to access aerial oxygen), as well as higher aerobic capacities (as indicated by citrate synthase activity), higher levels of lactate and lower levels of creatine phosphate in the muscle at the end of an encounter. In contrast, the capacity for glycolysis and anaerobic metabolism (as indicated by pyruvate kinase and lactate dehydrogenase activities) did not vary with aggression. This suggests that a high capacity for oxidative phosphorylation, either during or in recovery from agonistic encounters, underlies aggressive behavior. Although more actively striking fish make greater use of anaerobic glycolysis, they do so only to a modest extent that is within a capacity that does not vary between individuals with different levels of aggression. Overall, the use of, and biochemical capacity for, aerobic metabolism appears to be an important correlate of fighting performance in *B. splendens*.

Introduction

Agonistic interactions between individuals within animal species can, over evolutionary time, accentuate phenotypic traits (West-Eberhard, 1983). These traits are often most conspicuously expressed among males (Emlen & Oring, 1977), although this is not always the case (Kvarnemo & Ahnesjo, 1996). Although external morphological characteristics may be most obvious, underlying metabolic phenotypes are important for supplying the energy needed to support agonistic interactions. Agonistic interactions entail a substantial metabolic cost, as reflected by increases in whole-animal aerobic metabolism and oxygen consumption rate (Metcalfe, Taylor & Thorpe, 1995; Hack, 1997; Grantner & Taborsky, 1998; Sneddon, Taylor & Huntingford, 1999; deCarvalho, Watson & Field, 2004; Briffa & Sneddon, 2007). In addition, the metabolic demand created by rapid and exhaustive bouts of activity during aggressive behavior may outstrip the capacity for aerobic metabolism and oxygen supply, leading to an increased reliance on anaerobic metabolism supported by substrate-level phosphorylation via creatine phosphate (CrP) and/or anaerobic glycolysis (Fernandez, Meunier-Salaün & Mormede, 1994; Neat, Taylor & Huntingford, 1998; Briffa & Elwood, 2005; Le François, Lamarre & Blier, 2005). Capacities for energy metabolism could therefore limit success in agonistic interactions and result in potentially detrimental consequences for fitness (Briffa & Sneddon, 2007). Despite the underlying importance of energy supply (Mowles, Cotton &

Briffa, 2009, 2010), few studies have examined the relationship between aggressive behaviors and metabolic capacity, especially with regard to the cellular biochemical machinery (Guderley & Couture, 2005; Guderley, 2009).

The Siamese fighting fish *Betta splendens*, a bimodally breathing freshwater teleost found in southeast Asia, is an ideal species to study the metabolic bases of aggressive behavior. As a naturally aggressive fish, their aggressive displays have likely been accentuated by artificial selection in domestic populations in order to sustain their popularity in the international pet trade (Karino & Someya, 2007). Aggressive displays include direct strikes, opercular flares and dorsal fin spread, all of which have been correlated with fighting success (Simpson, 1968; Castro, Ros & Becker, 2006). To date, four studies have addressed various facets of the potential physiological and biochemical bases of aggression in *B. splendens* (Haller, 1991*a,b*; Castro *et al*., 2006; Alton, Portugal & White, 2013). Haller (1991*a,b*) found that direct fighting between cohabitating males caused depletion of glycogen, lipid and amino acid fuel sources. Castro *et al*. (2006) and Alton *et al*. (2013) both observed whole-animal oxygen consumption rate to increase by a greater magnitude in more aggressive males, either during or in recovery from paired encounters (oxygen consumption increased almost entirely due to aerial respiration during fights rather than aquatic respiration in recovery). Both Haller (1991*b*) and Alton *et al*. (2013) concluded that anaerobic glycolysis plays an important role for energy supply during aggressive encounters, which incurs an oxygen debt that must later be repaid through aerobic routes of energy production. This may result in part because opercular flaring dominates agonistic encounters, which should decrease the capacity for gill ventilation and the time available to breathe air and acquire oxygen at the surface (Abrahams, Robb & Hare, 2005). If so, the capacity for anaerobic metabolism should be an important determinant of fighting performance, but studies examining the relationship between the capacity for, or reliance on, anaerobically derived energy supply and the outcome of paired agonistic encounters are scarce.

We assayed key metabolites and enzymes of aerobic and anaerobic metabolism from post-fight male *B. splendens* to test whether variation in aggression is correlated with metabolic responses at the biochemical level. We used behavioral assays to quantify aggressive behavior and, relying on previous studies (Braddock & Braddock, 1955; Simpson, 1968), estimated the likelihood of an individual winning an agonistic encounter. In the muscle of the immediately post-aggressive individuals, we measured concentrations of CrP and lactate to estimate reliance on anaerobic pathways, as well as the activities of a glycolytic enzyme (pyruvate kinase; PK), an anaerobic glycolytic enzyme (lactate dehydrogenase; LDH), and an oxidative enzyme (citrate synthase; CS) to estimate how aggressive behavior relates to the biochemical capacities for anaerobic and aerobic energy production. We also measured whole-blood hemoglobin (Hb) concentration to determine if the oxygen-carrying capacity of the blood was different between individuals. Finally, we measured intracellular pH (pHi) and adenylate status to assess whether aggressive behavior is related to the ability to maintain acid-base balance, which can be perturbed by reliance upon anaerobic glycolysis or to maintain stable cellular energy levels, which can be perturbed by uncompensated metabolic demand for adenosine triphosphate (ATP). We predicted that if aerobic and anaerobic biochemical capacities are important determinants of aggressive behaviors, then these characteristics will covary with the behavioral parameters measured.

Materials and methods

Animals

Sixteen male Siamese fighting fish *Betta splendens* (average mass 1.47 ± 0.19 g) were obtained from a local supplier (Big Al's Aquarium Supercentres, Woodbridge, ON, Canada) and held individually in 500 mL vessels at 21°C and under a 12 h light/12 h dark photoperiod for 1 week prior to experiments. Water was Hamilton dechlorinated tap water originating from Lake Ontario (pH 8.0, Na^+ 13.8 mg L^{-1}, Cl^- 24.8 mg L^{-1}). Fish were fed with commercially available flake food (Big Al's Aquarium Supercentres) twice per day and 50% water changes were also conducted twice per day. Feeding was halted 24 h prior to the behavioral trials. Opaque partitions were placed between holding vessels to ensure that fish were visually isolated from one another throughout the holding period. All procedures followed guidelines established by the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board.

Behavioral experiments

Aggression was quantified using a size-matched pair of male fish (average mass difference per pair of 0.091 ± 0.080 g) placed on either side of a transparent pane of glass in a 20-L tank. To minimize handling stress, fish were transferred between holding and experimental tanks without removing them from water and a new volume of water (20.7 ± 1.1°C) was used for each individual experiment. Following a 20-min habituation period where an opaque divider shielded each fish's view of the other (although there was modest potential for chemical signals to travel between sides of the tank during the holding period), the opaque divider was removed and the two fish were allowed to perform aggressive behaviors across the transparent pane of glass for 20 min. At the end of each paired trial, fish were euthanized with a sharp blow to the head followed by pithing and a sample of the axial swimming muscle (including the entirety of the red and white musculature in a cross section) was freeze clamped in liquid N_2 and stored at –80°C. A small sample (6 μL) of blood was taken from the severed tail to measure whole-blood Hb content (using Drabkin's reagent following manufacturer instructions; Sigma-Aldrich, St. Louis, MO, USA).

The frequency and duration of two distinct motor patterns performed by the agonistic male *B. splendens* were used to quantify aggression and determine the outcome of their agonistic interactions (Simpson, 1968): (1) strikes, defined as aggressive bites toward the glass divider in the direction of the

fish's opponent that commenced with a C-start (a strike or escape reflex used by fishes to achieve a high acceleration; Domenici & Blake, 1997), differentiated from non-aggressive nudges against the glass with no attempted bite or C-start; (2) opercular flaring, including both total number and duration. Surfacing frequency, defined as non-aggressive movements to the surface to gulp air, was also scored. All behaviors were directly recorded by two observers randomly assigned to one of the two fish.

Strike frequency during the paired encounters was used as the metric for estimating the winner of each dyad. Although this is a slight contrast to the recent convention of attributing a winner in *B. splendens* pairings based on the frequency of opercular flaring (e.g. Castro *et al.*, 2006), it was our preferred metric for four reasons: (1) a strike would presumably result in a bite, which would have real consequences for the opponent fish (as opposed to 'challenging' behavior like opercular flaring); (2) striking and biting are the behaviors that tend to precipitate full-blown fights in *B. splendens* (Braddock & Braddock, 1955); (3) in male *B. splendens* pairings allowed to fight to completion, the winning fish has been shown to bite its opponent twice as often as the losing fish (Braddock & Braddock, 1955); (4) the greatest behavioral variation among the two individuals comprising the average dyad in our study was for strikes (Fig. 1a). Given this, we analyzed our data in two ways: first, as a categorical variable, where the fish were defined as either 'winners' or 'losers' based on strike frequency during the divider trials; and second, as a continuous variable, where the various characteristics (behavioral or biochemical) were correlated with strike frequency. Both observers were consistent with their quantification of strike frequency and there was no evidence that the randomly assigned observers recorded strike frequency differently ($P = 0.20$).

Enzyme and metabolite analyses

Frozen muscle tissue (40–60 mg) was ground using an insulated mortar and pestle cooled with liquid N_2 and was partitioned for enzyme or metabolite analyses. For measurement of enzyme activity, the ground tissue was homogenized on ice in 20 volumes of homogenization buffer (50 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 mmol L^{-1} ethylenediaminetetraacetic acid and 0.1% Triton X-100, pH 7.4 at 20°C) using a tenbroeck tissue grinder (Wheaton Industries, Millville, NJ, USA). Homogenates were centrifuged at 10 000× ***g*** for 2 min (4°C) and dilutions of the supernatant were used for the enzyme assays. We assayed three enzymes that reflect the capacity for aerobic and anaerobic energy metabolism: CS, PK and LDH. CS activity reflects the flux capacity of the tricarboxylic acid cycle and is thought to be a good metric of tissue mitochondrial density (Larsen *et al.*, 2012), and subsequently, aerobic capacity. PK is an indicator of the flux rate through glycolysis, while LDH is an indicator of the fish's capacity to produce ATP via anaerobic glycolysis (Pelletier *et al.*, 1995). Maximal enzyme activities (V_{max}) were assayed at 25°C by following the oxidation of NADH at 340 nm (LDH, PK) or the reaction between dithionitrobenzoic acid (DTNB) and liberated coenzyme A (CoA) at 412 nm (CS)

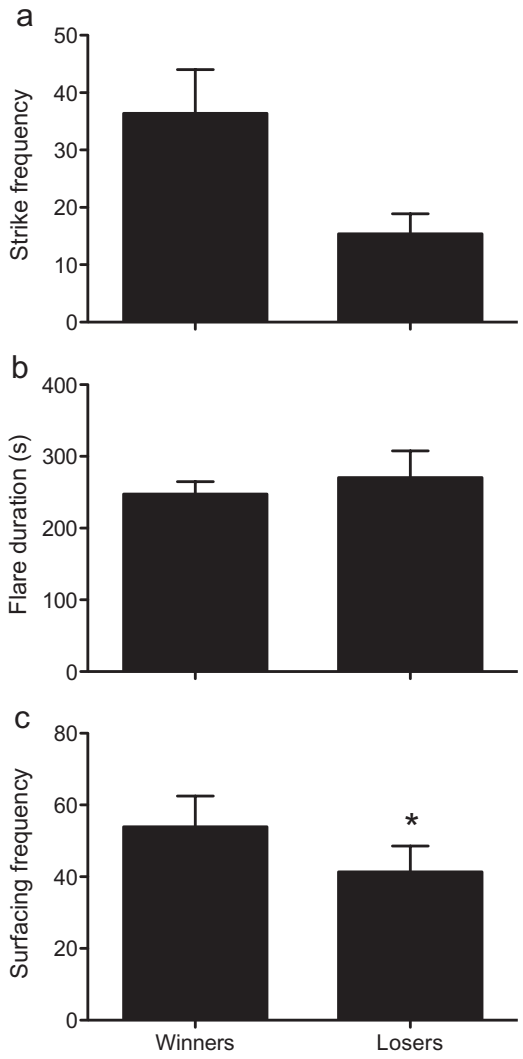

Figure 1 (a) Strike frequency, (b) flare duration (s) and (c) surfacing frequency by male *Betta splendens* when exposed to another size-matched male across a transparent divider for 20 min. 'Winners' and 'losers' were determined by strike frequency. The asterisk (*) indicates significant difference ($P < 0.05$) between winners and losers.

in a 96-well microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Assay conditions followed protocols that are well established for fish (e.g. Suarez, Mallet & Daxboeck, 1986; Moon & Mommsen, 1987): CS, 0.5 mmol L^{-1} oxaloacetate (omitted for control), 0.3 mmol L^{-1} acetyl-CoA and 0.1 mmol L^{-1} DTNB in 50 mmol L^{-1} tris(hydroxymethyl)aminomethane (pH 8.0); LDH, 1 mmol L^{-1} pyruvate (omitted for control) and 0.15 mmol L^{-1} NADH in 50 mmol L^{-1} HEPES (pH 7.4); PK, 5 mmol L^{-1} phosphoenolpyruvate (omitted for control), 0.15 mmol L^{-1} NADH, 5 mmol L^{-1} adenosine diphosphate (ADP), 100 mmol L^{-1} KCl, 10 mmol L^{-1} $MgCl_2$, 10 μmol L^{-1} fructose-1,6-bisphosphate and excess coupling enzyme (LDH) in 50 mmol L^{-1} 3-(*N*-morpholino)propanesulfonic acid (pH 7.4). Each reaction contained 10 μL of diluted homogenate in a final

well volume of 200 μL.Preliminary assays confirmed that saturating substrate concentrations were used and that enzyme reaction rates were proportional to the protein added. We used an extinction coefficient (ε) of 13.6 optical density (mmol L^{-1}) cm^{-1} for DTNB in the CS assay and we calculated ε for NADH empirically in the LDH and PK assays by constructing standard curves of absorbance versus NADH concentration in the buffers appropriate for each assay. Each sample was assayed in triplicate and activities were determined by subtracting the background (control) reaction rate without substrate from the rates measured in the presence of substrate.

We also measured the metabolite concentrations for lactate, ATP, CrP and creatine (Cr). Lactate is the end product of anaerobic glycolysis and is produced in proportion to the amount of ATP generated by anaerobic glycolysis. CrP is used for rapid, oxygen-independent ATP production in which a phosphate group is transferred directly from CrP to ADP via creatine kinase (CK), yielding ATP and free Cr (Cr_{free}). Ground frozen muscle of *c*. 25 mg was sonicated (three 3-s passes) in 10 volumes of a metabolic inhibitor solution consisting of 150 mmol L^{-1} potassium fluoride and 6 mmol L^{-1} nitrilotriacetic acid (Pörtner, 1990). pHi was measured in an aliquot of this homogenate using an Accumet microcombination pH electrode (Fisher Scientific model 13-620-96; Fisher Scientific, Hampton, NH, USA). The remaining homogenate, to be used for metabolite measurements, was acidified in 30% perchloric acid, neutralized in 3 M potassium carbonate, centrifuged at 10 000× ***g*** for 10 min (4°C) and then stored at –80°C. Upon thawing, these samples were centrifuged again at 10 000× ***g*** for 10 min (4°C) immediately prior to metabolite measurements. ATP and CrP concentrations were analyzed using the coupled CK, hexokinase and glucose 6-phosphate dehydrogenase reactions as described by Bergmeyer (1983). Cr_{free} concentrations were measured with the coupling enzymes CK, PK and LDH according to Bergmeyer (1983). Finally, lactate concentrations were measured with LDH according to Bergmeyer (1983).

We expressed all enzyme activities and metabolite contents per gram of wet tissue in order to assess the overall biochemical capacity and status of the tissue. Standardizing our measurements to mg protein could discount the contributions of variable water content or protein content to biochemical capacity of the tissue and there was no *a priori* reason to discount these contributions in addressing how tissue biochemical capacity relates to fighting performance.

Calculations and statistics

Measured levels of [ATP], [CrP], [Cr] and pHi were used to calculate free cytosolic [ADP], assuming equilibrium of the CK reaction:

$$[ADP_{free}] = \frac{[ATP][Cr]}{[CrP]K'_{CK}}$$

The equilibrium constants for CK (K'_{CK}) were adjusted to account for pHi and acclimation temperature (Golding, Teague & Dobson, 1995; Teague, Golding & Dobson, 1996). Free Mg^{2+} was assumed to be 1 mmol L^{-1} (Van Waarde *et al.*, 1990; Jibb & Richards, 2008).

To compare behavioral and biochemical characteristics between winners and losers, we used a paired *t*-test in R 3.1.2 (R Core Team, 2013). In cases where the data were not normally distributed, we used a paired Wilcoxon signed rank test. We used a paired test in this case as it is important to consider the possibility of non-independence between pairs, where the two fish within a dyad could influence each other's behavior. To compare the correlation of these characteristics with strike frequency, we used a Pearson correlation, also in R.

Results

Behavioral experiments

Across the agonistic pairings, the fish that struck more frequently (i.e. winners) did so on average 35.9 times compared with 15.4 for the low-striking fish (i.e. losers; Fig. 1a). Therefore, the outcome of the trials was decisive: within a dyad, the fish differed by an average of 21.0 strikes (SEM = 5.86, $n = 8$). In addition, striking was continuous and evenly distributed across time throughout the 20-min trials for both winners and losers (Supporting Information Fig. S1). Total flare number did not differ between winners and losers (40.5 vs. 42.5, $P = 0.80$; data not shown) nor did total flare duration (Fig. 1b), which also did not covary with strike frequency ($r = 0.05$, $P = 0.41$). However, we found that average flare frequency was significantly and positively correlated with strike frequency ($r = 0.53$, $P = 0.04$). There was also a significant relationship between strike frequency and surfacing frequency ($r = 0.67$, $P = 0.005$; Fig. 2) and winners surfaced significantly more than losers (Fig. 1c).

Biochemical results: enzyme activities and metabolites

The fish that struck more frequently during the agonistic encounters had significantly higher CS activities, an aerobic

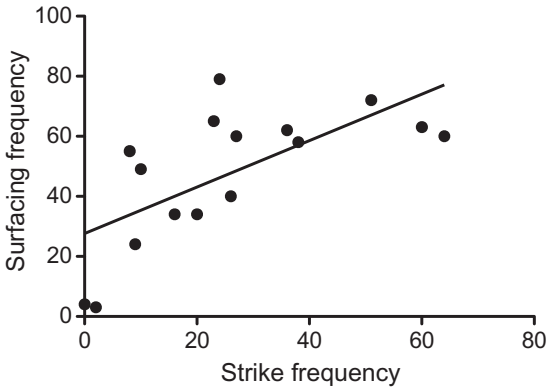

Figure 2 Positive relationship between surfacing frequency and strike frequency ($r = 0.67$, $P = 0.005$) by male *Betta splendens* when exposed to another size-matched male across a transparent divider for 20 min.

Table 1 Citrate synthase (CS), lactate dehydrogenase (LDH) and pyruvate kinase (PK) enzyme activities (μmol min^{-1} g^{-1} wet tissue) in the muscle of male *Betta splendens* exposed to another size-matched male across a transparent divider for 20 min. 'Winners' and 'losers' were determined by strike frequency

Measure	Winners	Losers	Effect size (*t*-test) and *P* value for comparison of winners and losers	Pearson correlation and *P* value with strike frequency
CS	2.22 ± 0.06	2.07 ± 0.04	$t = -2.0$, $P = 0.09$	$r = 0.49$, $P = 0.05$
LDH	201.3 ± 5.41	207.4 ± 10.2	$t = -0.55$, $P = 0.60$	$r = 0.05$, $P = 0.85$
PK	110.1 ± 4.73	114.2 ± 5.66	$t = -0.55$, $P = 0.59$	$r = -0.09$, $P = 0.75$

Data are expressed as means ± SEM ($n = 8$).

enzyme, than the fish that struck less frequently ($r = 0.49$, $P = 0.05$; Table 1, Fig. 3a) and the winning fish tended to have higher activity of CS in the axial locomotory muscle compared with the losing fish ($t = -1.97$, $P = 0.09$; Table 1). In contrast, the activities of LDH and PK, which are involved in anaerobic glycolysis, did not differ significantly between winners and losers, nor did they correlate to strike frequency (Table 1).

[CrP] was significantly negatively correlated with strike frequency ($r = -0.53$, $P = 0.03$; Table 2, Fig. 3b) although there was not a strong difference between winners and losers ($t = -1.71$, $P = 0.13$; Table 2). The size of the total Cr pool [Cr_{total}, the sum of (CrP) and (Cr_{free})] did not differ between winners and losers and did not correlate significantly with strike frequency, but there was a trend for negative association between these variables (Table 2). No variation in [Cr_{free}] content was observed. Muscle lactate levels were strongly positively correlated with striking frequency ($r = 0.78$, $P = 0.0004$; Fig. 3c), but there were no significant differences in muscle pHi, [ATP] or [ADP_{free}] observed between the winners and losers of agonistic pairings nor were there significant associations of these variables with strike frequency (Table 2).

Finally, no significant differences were observed for whole-blood [Hb] between winners and losers (Table 2), nor when these concentrations were expressed as a function of strike frequency (Table 2), suggesting that the circulatory oxygen-carrying capacity did not vary with aggression.

Discussion

Biochemical capacities can have significant implications for physiological performance, social behavior, predator avoidance, resource acquisition and aggression (Briffa & Sneddon, 2007). Here, we have provided a detailed test of whether variation in aerobic and/or anaerobic metabolism is correlated with aggressive behavior in *B. splendens*, a useful organism for studying the physiological bases of aggressive behaviors (Haller, 1991*a,b*; Castro *et al.*, 2006; Alton *et al.*, 2013). We found that fish that exhibited a greater proclivity for striking a conspecific were more likely to surface for air, which agrees with the previous findings of elevated oxygen consumption rate and air-breathing frequency in winning fish during a fight (Alton *et al.*, 2013). Consistent with a major role for aerobic metabolism in sustained aggression, we found that strike frequency was positively correlated with CS activity. In contrast, our data provide no evidence that greater aggression is associated with a greater *capacity* for anaerobic ATP generation because LDH and PK enzyme activities were not related to aggression nor were they different between winners and losers. However, higher aggression requires a greater realized *use* of anaerobic metabolism, within an invariant capacity of these pathways, because greater strike frequency was associated with higher [lactate] and lower [CrP] in the muscle (Fig. 3). Our results suggest that both aerobic and anaerobic energy supply help maintain cellular [ATP] (which were preserved similarly in all fish after aggressive encounters) and contribute to success in agonistic interactions.

Frequent strikers may benefit from a higher aerobic capacity for ATP production to maintain muscle [ATP] via oxidative phosphorylation based on the positive correlation between CS activity and strike frequency. CS is often used as an indicator of mitochondrial density (e.g. Larsen *et al.*, 2012), and subsequently, aerobic capacity. If this enhanced capacity is utilized in the winning fish, then it is not driven by a disruption of cytosolic adenylates – which are known to stimulate oxidative phosphorylation (Brand & Murphy, 1987; Cieslar, 2000) – because there were no significant differences in [ADP_{free}] or [ADP_{free}] : [ATP] (see Bessman & Carpenter, 1985 for review). The muscle samples taken in the present study were taken from mixed tissue, so the variation in muscle CS activity could be associated with parallel variation in the density of oxidative muscle fibers. Should these attributes provide the fish with a greater buffer against ATP loss, they may allow an aggressor to strike with more abandon.

The importance of aerobic respiration in agonistic encounters is supported by the strong positive relationship between striking frequency and surfacing frequency (to breathe air). We also found a significant relationship between surfacing and flare frequencies, although it was not as strong as observed with strike frequency. These findings suggest that striking and displaying fish require more oxygen, which is consistent with the results of previous studies (Dore, Lefebvre & Ducharme, 1978; Alton *et al.*, 2013). In particular, Alton *et al.* (2013) found that *B. splendens* – a facultative air breather – more than doubles oxygen consumption during agonistic encounters almost exclusively via increased aerial respiration. Interestingly, increasing scope for aerobic metabolism has been proposed as a likely driver for the repeated evolution of air-breathing in warm, hypoxic waters typical of *B. splendens* habitat (Giomi *et al.*, 2014). Thus, selection for agonistic

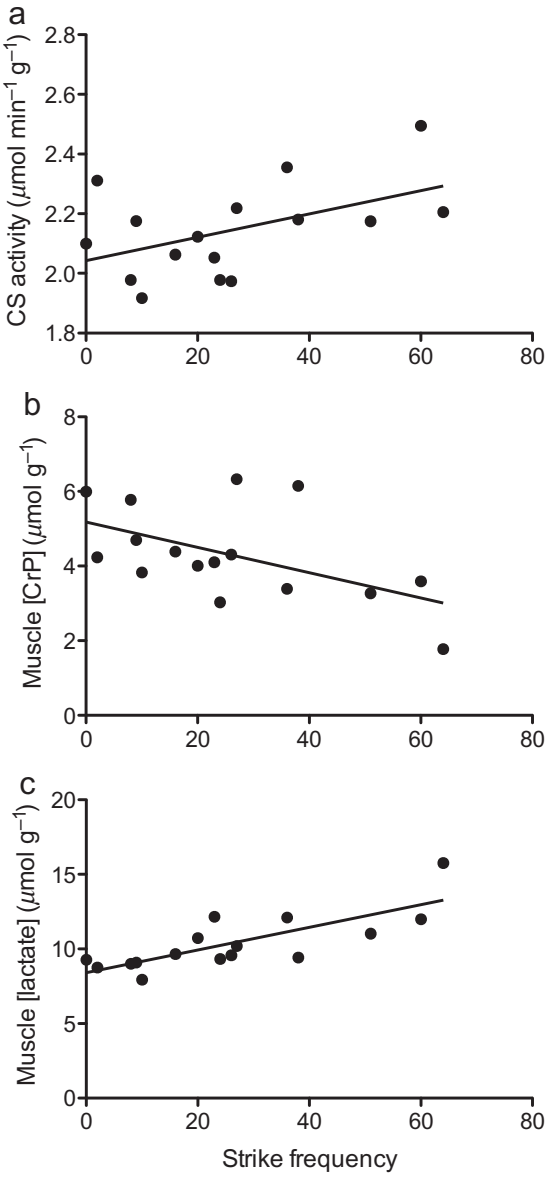

Figure 3 Relationship between (a) muscle citrate synthase (CS) activity and strike frequency ($r = 0.49$, $P = 0.05$), (b) muscle [creatine phosphate, CrP] and strike frequency ($r = -0.53$, $P = 0.03$) and (c) muscle [lactate] and strike frequency ($r = 0.78$, $P = 0.0004$) by male *Betta splendens* when exposed to another size-matched male across a transparent divider for 20 min. All units are expressed per gram of wet tissue.

performance in *B. splendens* might have enhanced selection for air-breathing, especially given that opercular flaring may reduce the capacity for aquatic respiration (Alton *et al.*, 2013).

We originally predicted that the capacity for and/or use of anaerobic ATP production pathways would be correlated with aggression due to the kinematic similarities of striking behavior and fast-starts (Domenici, 2011). Potentially consistent with there being a greater use of substrate-level phosphorylation via the CK reaction, muscle [CrP] was negatively correlated with strike frequency. However, there was no significant difference in [Cr_{free}] in the muscle of winning and losing fish nor was [Cr_{free}] correlated with strike frequency (Table 2). A positive correlation between [Cr_{free}] and strike frequency would have also been expected if there was a greater use of CrP by the more aggressive fish. It is instead possible that the negative correlation between [CrP] and strike frequency was an indirect consequence of variation in muscle aerobic capacity. For example, if winners had greater CS activity because they had more oxidative muscle fibers (and thus fewer glycolytic muscle fibers), then winners would be expected to have a lower total Cr pool [(CrP) + (Cr_{free})] because these metabolites are known to be more abundant in white muscle than in red muscle (Dunn & Hochachka, 1986). Indeed, our results show that there is a trend for those fish with a greater proclivity to strike to have a lower total Cr pool (Table 2).

Consistent with there being an association between aggressive behavior and the use of anaerobic glycolysis, muscle [lactate] was positively correlated with strike frequency (Fig. 3c). Even though fish that struck more often accumulated more of the primary end product of anaerobic glycolysis, they did not develop a metabolic acidosis (Table 2). However, the absolute difference in accumulated lactate between the highest-striking and lowest-striking fish was not large and there was no difference in [lactate] between winners and losers (Table 2). Furthermore, the amount of lactate accumulated by the high-striking fish in this study (11.0 ± 0.81 μmol g^{-1}) is comparatively low when compared with the high levels of lactate that accumulate in the muscle of exhaustively exercised fish [e.g. ~107 μmol g^{-1} in skipjack tuna (Arthur *et al.*, 1992); ~50 μmol g^{-1} in rainbow trout (Milligan & Wood, 1986); ~25 μmol g^{-1} in lamprey (Boutilier *et al.*, 1993); ~24 μmol g^{-1} in yellow perch (Schwalme & Mackay, 1991)]. Thus, aggressive behavior of *B. splendens* may be only modestly reliant on energy supply via anaerobic glycolysis. Consistent with this, anaerobic capacities in muscle, as reflected by PK and LDH activities, were not associated with aggressive behavior (Table 1). Nevertheless, the PK activities in the axial muscle (the entirety of one hemisphere of red and white fibers) of *B. splendens* from our study (110–114 μmol min^{-1} g^{-1}) are high compared with PK activities in the axial muscle of other similar sized fish species assayed at the same approximate temperature [~40–50 μmol min^{-1} g^{-1} for *Danio rerio* (Schnurr, Yin & Scott, 2014); 12.4–50.2 μmol min^{-1} g^{-1} for white muscle PK activity of 12 sculpin species from the family Cottidae (Mandic, Speers-Roesch & Richards, 2013); 105 μmol min^{-1} g^{-1} for *Fundulus grandis* and 87.1 μmol min^{-1} g^{-1} for *Fundulus similis* (Pierce & Crawford, 1997); 109.5 μmol min^{-1} g^{-1} for *Salmo gairdneri* (Knox, Walton & Cowey, 1980)], so *B. splendens* muscle appears to have a comparatively high glycolytic capacity. The muscle LDH activities of *B. splendens* tend to be less than those of these same comparative species, however, suggesting that the

Table 2 [ATP], [Cr_{free}], [CrP], [Cr_{total}], pHi, [Hb], [ADP_{free}] and [lactate] in the muscle of male *Betta splendens* exposed to another size-matched male across a transparent divider for 20 min. 'Winners' and 'losers' were determined by strike frequency

Measure	Winners	Losers	Effect size (*t*-test) and *P* value for comparison of winners and losers	Pearson correlation and *P* value with strike frequency
[ATP]	0.20 ± 0.04	0.24 ± 0.03	$t = -1.23$, $P = 0.48$	$r = -0.02$, $P = 0.93$
[Cr_{free}]	18.0 ± 0.54	18.1 ± 0.65	$t = -0.08$, $P = 0.94$	$r = -0.13$, $P = 0.64$
[CrP]	3.73 ± 0.45	4.88 ± 0.35	$t = -1.7$, $P = 0.13$	$r = -0.53$, $P = 0.03$
[Cr_{total}]	21.7 ± 0.65	22.9 ± 0.73	$t = -1.5$, $P = 0.18$	$r = -0.44$, $P = 0.09$
pHi	6.61 ± 0.07	6.67 ± 0.05	$t = -0.56$, $P = 0.59$	$r = -0.41$, $P = 0.11$
[Hb]	10.9 ± 1.04	10.7 ± 0.84	$t = 0.89$, $P = 0.41$	$r = 0.26$, $P = 0.35$
[ADP_{free}]	1.74 ± 0.35	1.89 ± 0.39	$t = -0.28$, $P = 0.79$	$r = 0.02$, $P = 0.94$
[lactate]	11.0 ± 0.81	9.74 ± 0.45	$t = 1.59$, $P = 0.16$	$r = 0.78$, $P = 0.0004$

Data are expressed as means ± SEM ($n = 8$). ADP, adenosine diphosphate; ATP, adenosine triphosphate; Cr, creatine; CrP, creatine phosphate; Hb, hemoglobin; pHi, intracellular pH. [ATP], [Cr_{free}], [CrP] and [lactate] are expressed in μmol g^{-1} wet tissue; [ADP_{free}] is expressed in nmol g^{-1} wet tissue. [Hb] is expressed in g dL^{-1}.

high glycolytic capacity of *B. splendens* serves a greater role in the production of pyruvate and reducing equivalents for oxidative phosphorylation (i.e. aerobic energy supply) than it does for the anaerobic supply of energy.

Conclusions

More aggressive male *B. splendens* have higher aerobic capacities in the locomotory muscle than less aggressive male *B. splendens* and have only a modestly greater use of – and no greater capacity for – anaerobic ATP production. Therefore, oxidative phosphorylation, rather than anaerobic metabolism, may fuel a greater proportion of muscle activity during aggressive encounters and may speed the recovery from muscle bursts associated with each episode of striking. Consistent with an enhanced aerobic capacity at the tissue level, more aggressive individuals also surfaced more frequently during confrontations, presumably to access aerial sources of oxygen and to help meet the greater oxygen demands of frequent striking. Altogether, our results suggest that the biochemical capacity for aerobic metabolism in *B. splendens* muscle is an important determinant of inter-individual variation in aggressive behavior, likely more important than the use of and capacity for anaerobic metabolism, and may be under selective pressure.

Acknowledgments

The authors would like to thank S.O. Stawski and Romanyk for their help to make this research possible and the two reviewers for their helpful and insightful comments. The equipment and operational costs of this research was supported by funds from McMaster University, the Canadian Foundation for Innovation, the Ontario Ministry of Research and Innovation and a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to G.R.S. B.S.R. was supported by an NSERC Postdoctoral Fellowship. M.D.R. was supported by an NSERC Postgraduate Scholarship and a University of British Columbia Zoology Graduate Fellowship. D.P.L.T. was supported by a UBC Zoology Graduate Fellowship. M.A.S. was supported by an NSERC Canada Graduate Scholarship. J.S.B. was supported by a DePaul University start-up grant.

Author contributions

All authors contributed to the experimental design, execution of experiments, experimental analyses and interpretation of data. BSR proposed the initial concept for the study and all authors helped develop it. MDR and DPLT wrote the first draft of the manuscript and all authors provided editorial input.

References

Abrahams, M.V., Robb, T.L. & Hare, J.F. (2005). Effect of hypoxia on opercular displays: evidence for an honest signal? *Anim. Behav.* **70**, 427–432.

Alton, L.A., Portugal, S.J. & White, C.R. (2013). Balancing the competing requirements of air-breathing and display behaviour during male–male interactions in Siamese fighting fish *Betta splendens*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **164**, 363–367.

Arthur, P.G., West, T.G., Brill, R.W., Schulte, P.M. & Hochachka, P.W. (1992). Recovery of skipjack tuna (*Katsuwonus pelamis*) white muscle: rapid and parallel changes in lactate and phosphocreatine after exercise. *Can. J. Zool.* **70**, 1230–1239.

Bergmeyer, H.U. (1983). *Methods of enzymatic analysis*. 3rd edn. Basel: Verlag Chemie.

Bessman, S.P. & Carpenter, C.L. (1985). The creatine-creatine phosphate energy shuttle. *Annu. Rev. Biochem.* **54**, 831–862.

Boutilier, R.G., Ferguson, R.A., Henry, R.P. & Tufts, B.L. (1993). Exhaustive exercise in the sea lamprey (*Petromyzon marinus*): relationship between anaerobic metabolism and intracellular acid-base balance. *J. Exp. Biol.* **178**, 71–88.

Braddock, J.C. & Braddock, Z.I. (1955). Aggressive behavior among females of the Siamese fighting fish, *Betta splendens*. *Physiol. Zool.* **28**, 152–172.

Brand, M.D. & Murphy, M.P. (1987). Control of electron flux through the respiratory chain in mitochondria and cells. *Biol. Rev. Camb. Philos. Soc.* **62**, 141–193.

Briffa, M. & Elwood, R.W. (2005). Rapid change in energy status in fighting animals: causes and effects of strategic decisions. *Anim. Behav.* **70**, 119–124.

Briffa, M. & Sneddon, L.U. (2007). Physiological constraints on contest behaviour. *Funct. Ecol.* **21**, 627–637.

Castro, N., Ros, A. & Becker, K. (2006). Metabolic costs of aggressive behaviour in the Siamese fighting fish, *Betta splendens*. *Aggress. Behav.* **32**, 474–480.

Cieslar, J.H. (2000). Free [ADP] and aerobic muscle work follow at least second order kinetics in rat gastrocnemius in vivo. *J. Biol. Chem.* **275**, 6129–6134.

deCarvalho, T.N., Watson, P.J. & Field, S.A. (2004). Costs increase as ritualized fighting progresses within and between phases in the sierra dome spider, *Neriene litigiosa*. *Anim. Behav.* **68**, 473–482.

Domenici, P. (2011). Fast start. In *Encyclopedia of fish physiology: buoyancy, locomotion and movement in fishes*: 587–596. Farrell, A.P. (Ed.). London: Academic Press.

Domenici, P. & Blake, R. (1997). The kinematics and performance of fish fast-start swimming. *J. Exp. Biol.* **200**, 1165–1178.

Dore, F., Lefebvre, L. & Ducharme, R. (1978). Threat display in *Betta splendens*: effects of water condition and type of agonistic stimulation. *Anim. Behav.* **26**, 738–745.

Dunn, J.F. & Hochachka, P.W. (1986). Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. *J. Exp. Biol.* **123**, 229–242.

Emlen, S.T. & Oring, L.W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science* **197**, 215–223.

Fernandez, X., Meunier-Salaün, M.C. & Mormede, P. (1994). Agonistic behavior, plasma stress hormones, and metabolites in response to dyadic encounters in domestic pigs: interrelationships and effect of dominance status. *Physiol. Behav.* **56**, 841–847.

Giomi, F., Fusi, M., Barausse, A., Mostert, B., Pörtner, H.O. & Cannicci, S. (2014). Improved heat tolerance in air drives the recurrent evolution of air-breathing. *Proc. Biol. Sci.* **281**, 1–8.

Golding, E.M., Teague, W.E. & Dobson, G.P. (1995). Adjustment of K′ to varying pH and pMg for the creatine kinase, adenylate kinase and ATP hydrolysis equilibria permitting quantitative bioenergetic assessment. *J. Exp. Biol.* **198**, 1775–1782.

Grantner, A. & Taborsky, M. (1998). The metabolic rates associated with resting, and with the performance of agonistic, submissive and digging behaviours in the cichlid fish *Neolamprologus pulcher* (Pisces: Cichlidae). *J. Comp. Physiol. [B.]* **168**, 427–433.

Guderley, H. (2009). Muscle metabolic capacities and plasma cortisol levels of the male three-spine stickleback *Gasterosteus aculeatus*: are there 'femme fatale' or 'macho male' effects? *Physiol. Biochem. Zool.* **82**, 653–661.

Guderley, H. & Couture, P. (2005). Stickleback fights: why do winners win? Influence of metabolic and morphometric parameters. *Physiol. Biochem. Zool.* **78**, 173–181.

Hack, M.A. (1997). The energetic costs of fighting in the house cricket, *Acheta domesticus*. *Behav. Ecol.* **8**, 28–36.

Haller, J. (1991a). Biochemical cost of a fight in fed and fasted *Betta splendens*. *Physiol. Behav.* **49**, 79–82.

Haller, J. (1991b). Muscle metabolic changes during the first six hours of cohabitation in pairs of male *Betta splendens*. *Physiol. Behav.* **49**, 1301–1303.

Jibb, L.A. & Richards, J.G. (2008). AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, *Carassius auratus*. *J. Exp. Biol.* **211**, 3111–3122.

Karino, K. & Someya, C. (2007). The influence of sex, line, and fight experience on aggressiveness of the Siamese fighting fish in intrasexual competition. *Behav. Process.* **75**, 283–289.

Knox, D., Walton, M.J. & Cowey, C.B. (1980). Distribution of enzymes of glycolysis and gluconeogenesis in fish tissues. *Mar. Biol.* **56**, 7–10.

Kvarnemo, C. & Ahnesjo, I. (1996). The dynamics of operational sex ratios and competition for mates. *Trends Ecol. Evol.* **11**, 404–408.

Larsen, S., Nielsen, J., Hansen, C.N., Nielsen, L.B., Wibrand, F., Stride, N., Schroder, H.D., Boushel, R., Helge, J.W., Dela, F. & Hey-Mogensen, M. (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol.* **590**, 3349–3360.

Le François, N.R., Lamarre, S.G. & Blier, P.U. (2005). Is white muscle anaerobic glycolysis capacity indicative of competitive ability in Arctic charr? *J. Fish Biol.* **66**, 1167–1176.

Mandic, M., Speers-Roesch, B. & Richards, J.G. (2013). Hypoxia tolerance in sculpins is associated with high anaerobic enzyme activity in brain but not in liver or muscle. *Physiol. Biochem. Zool.* **86**, 92–105.

Metcalfe, N.B., Taylor, A.C. & Thorpe, J.E. (1995). Metabolic rate, social status and life-history strategies in Atlantic salmon. *Anim. Behav.* **49**, 431–436.

Milligan, C.L. & Wood, C.W. (1986). Tissue intracellular acid-base status and the fate of lactate after exhaustive exercise in the rainbow trout. *J. Exp. Biol.* **123**, 123–144.

Moon, T.W. & Mommsen, T.P. (1987). Enzymes of intermediary metabolism in tissue of the little skate, *Raja erinacea*. *J. Exp. Zool.* **244**, 9–15.

Mowles, S.L., Cotton, P.A. & Briffa, M. (2009). Aerobic capacity influences giving-up decisions in fighting hermit crabs: does stamina constrain contests? *Anim. Behav.* **78**, 735–740.

Mowles, S.L., Cotton, P.A. & Briffa, M. (2010). Whole organism performance capacity predicts resource-holding potential in the hermit crab *Pagurus bernhardus*. *Anim. Behav.* **80**, 277–282.

Neat, F., Taylor, A. & Huntingford, F. (1998). Proximate costs of fighting in male cichlid fish: the role of injuries and energy metabolism. *Anim. Behav.* **55**, 875–882.

Pelletier, D., Blier, P., Dutil, J. & Guderley, H. (1995). How should enzyme activities be used in fish growth studies? *J. Exp. Biol.* **198**, 1493–1497.

Pierce, V.A. & Crawford, D.L. (1997). Phylogenetic analysis of thermal acclimation of the glycolytic enzymes in the genus *Fundulus*. *Physiol. Zool.* **70**, 597–609.

Pörtner, H.O. (1990). Determination of intracellular buffer values after metabolic inhibition by fluoride and nitrilotriacetic acid. *Respir. Physiol.* **81**, 275–288.

R Core Team. (2013). *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.

Schnurr, M.E., Yin, Y. & Scott, G.R. (2014). Temperature during embryonic development has persistent effects on metabolic enzymes in the muscle of zebrafish. *J. Exp. Biol.* **217**, 1370–1380.

Schwalme, K. & Mackay, W.C. (1991). Mechanisms that elevate the glucose concentration of muscle and liver in yellow perch (*Perca flavescens* Mitchill) after exercise-handling stress. *Can. J. Zool.* **69**, 456–461.

Simpson, M.J.A. (1968). The display of the Siamese fighting fish, *Betta splendens*. *Anim. Behav.* **1**, 1–73.

Sneddon, L., Taylor, A. & Huntingford, F. (1999). Metabolic consequences of agonistic behaviour: crab fights in declining oxygen tensions. *Anim. Behav.* **57**, 353–363.

Suarez, R.K., Mallet, M.D. & Daxboeck, C. (1986). Enzymes of energy metabolism and gluconeogenesis in the Pacific blue marlin, *Makaira nigricans*. *Can. J. Zool.* **64**, 694–697.

Teague, W.E., Golding, E.M. & Dobson, G.P. (1996). Adjustment of K′ for the creatine kinase, adenylate kinase and ATP hydrolysis equilibria to varying temperature and ionic strength. *J. Exp. Biol.* **199**, 509–512.

Van Waarde, A., Van Den Thillart, G., Erkelens, C., Addink, A. & Lugtenburg, J. (1990). Functional coupling of glycolysis and phosphocreatine utilization in anoxic fish muscle: an *in vivo* 31P NMR study. *J. Biol. Chem.* **256**, 914–923.

West-Eberhard, M.J. (1983). Sexual selection, social competition, and speciation. *Q. Rev. Biol.* **58**, 155–183.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. The cumulative number of strikes over time for four randomly chosen trials. The steady increase in strikes over time illustrates that fish strike throughout the trials and there is little evidence of delays or declining strike intensity over time. In addition, individuals identified as winners at the end of the 10-min trial have higher cumulative strikes over the entire trial (i.e. running the trials for only 5 min would, in most cases, result in the same winners).