The Journal of Experimental Biology 215, 1847-1853

doi:10.1242/jeb.067918

RESEARCH ARTICLE

How well do muscle biomechanics predict whole-animal locomotor performance? The role of Ca^{2+} handling

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Accepted 11 February 2012

SUMMARY

It is important to determine the enabling mechanisms that underlie locomotor performance to explain the evolutionary patterns and ecological success of animals. Our aim was to determine the extent to which calcium (Ca^{2+}) handling dynamics modulate the contractile properties of isolated skeletal muscle, and whether the effects of changing Ca^{2+} handling dynamics in skeletal muscle are paralleled by changes in whole-animal sprint and sustained swimming performance. Carp (*Cyprinus carpio*) increased swimming speed by concomitant increases in tail-beat amplitude and frequency. Reducing Ca^{2+} release from the sarcoplasmic reticulum (SR) by blocking ryanodine receptors with dantrolene decreased isolated peak muscle force and was paralleled by a decrease in tail-beat frequency and whole-animal sprint performance. An increase in fatigue resistance following dantrolene treatment may reflect the reduced depletion of Ca^{2+} stores in the SR associated with lower ryanodine receptor (RyR) activity. Blocking RyRs may be detrimental by reducing force production and beneficial by reducing SR Ca^{2+} depletion so that there was no net effect on critical sustained swimming speed (U_{crit}). In isolated muscle, there was no negative effect on force production of blocking Ca^{2+} release *via* dihydropyridine receptors (DHPRs) with nifedipine. Nifedipine decreased fatigue resistance of isolated muscle, which was paralleled by decreases in tail-beat frequency and U_{crit}. However, sprint performance also decreased with DHPR inhibition, which may indicate a role in muscle contraction of the Ca^{2+} released by DHPR into the myocyte. Inhibiting sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) activity with thapsigargin decreased fatigue resistance, suggesting that SERCA activity is important in avoiding Ca^{2+} store depletion and fatigue. We have shown that different molecular mechanisms modulate the same muscle and whole-animal traits, which provides an explanatory model for the observed variations in locomotor performance within and between species.

Key words: sprint performance, fatigue resistance, ryanodine receptor, dihydropyridine receptor, swimming kinematics, SERCA.

INTRODUCTION

Locomotor performance represents the non-cognitive dimension of animal behaviour and is important during intraspecific and interspecific interactions between individuals. Absolute performance capacity, such as increased fatigue resistance, influences ecological success and fitness by increasing success during escape from predators (Walker et al., 2005; Langerhans, 2009), in competitive aggressive interactions (Garland et al., 1990; Husak et al., 2006; Wilson et al., 2009) and in acquisition of resources (Bennett, 1991). In addition, the relative capacity for sprint and endurance performance is linked to behavioural modes such as pursuit *versus* sit-and-wait predation (Perry, 1999), sneaky mating *versus* prolonged courtship behaviour (Wilson et al., 2010) and even sporting success in long-distance *versus* sprint or strength disciplines (Van Damme et al., 2002). Selection acts on whole-animal traits such as sprint or endurance locomotor performance. However, the evolutionary process itself occurs at the level of genetic and molecular mechanisms (Lande and Arnold, 1983). Hence, sub-organismal traits are often used to explain the success of animals in particular environments or circumstances, and to predict the vulnerability of species to change (Gibb and Dickson, 2002; James et al., 2007; Claireaux and Lefrançois, 2007). However, the relationship between sub-organismal traits and whole-animal performance remains unresolved (Gibb and Dickson, 2002). It is important to determine the enabling mechanisms that underlie locomotor performance to explain evolutionary patterns by revealing the traits that selection may act upon (Arnold, 1983; Irschick and Garland, 2001; Irschick et al., 2008), and to assess constraints to locomotor performance (Dickinson et al., 2000; Le Galliard et al., 2004). The aim of this research was therefore to determine the extent to which calcium (Ca^{2+}) handling dynamics modulate the contractile properties of isolated skeletal muscle, and whether the effects of changing calcium handling dynamics in skeletal muscle are paralleled by changes in whole-animal sprint and sustained swimming performance.

Locomotor performance depends principally on muscle performance, at least in species that have broadly similar morphological configurations (Vanhooydonck et al., 2001). Muscle performance depends on a number of extrinsic factors such as oxygen and nutrient supply by the cardiovascular system (Joyner and Coyle, 2008; Steinhausen et al., 2008), and intrinsic factors such as cellular metabolic capacities and myosin ATPase activities (Johnston et al., 1985; Johnson and Bennett, 1995; Johnston and Temple, 2002; Glanville and Seebacher, 2006), and the efficacy of Ca^{2+} handling dynamics (Berchtold et al., 2000; James et al., 2011). Ca^{2+} handling dynamics within the muscle cell are one of the most

important intrinsic determinants of muscle performance (Berchtold et al., 2000). Differences in expression and activity of the Ca^{2+} release channels dihydropyridine receptor (DHPR) and ryanodine receptor (RyR), and Ca^{2+} re-sequestration by sarco(endo)plasmic reticulum ATPase (SERCA) can affect power output and fatigue resistance of isolated muscle (Fleming et al., 1990; Syme and Tonks, 2004; James et al., 2011). However, the impact of these molecules on whole-animal performance remains unresolved, although the increase in densities of DHPR and RyR with endurance training in some fish (Antilla et al., 2006) indicates that these molecules are important for whole-animal locomotion.

We tested the following hypotheses. (1) Inhibition of DHPR-mediated Ca^{2+} flow across the sarcolemma will have a limited effect on isolated muscle mechanics and sprint performance, but it will influence sustained swimming performance by its effect on the cardiovascular system. DHPR are voltage-gated Ca^{2+} channels, which, upon neural stimulation, release Ca^{2+} into the muscle cell and stimulate RyR (Meissner, 1994). In cardiac muscle, RyR activation is dependent on Ca^{2+} release by DHPR, whereas in skeletal muscle RyR is activated by direct interaction with DHPR, independent of Ca^{2+} release (Dirksen, 2009). (2) Slowing release of Ca^{2+} from the sarcoplasmic reticulum (SR) by blocking RyR will slow muscle contraction and decrease force production. The Ca^{2+} released into the muscle cell from the SR binds to troponin and thereby facilitates interaction between actin and myosin to cause muscle contraction (Berchtold et al., 2000). The rate of Ca^{2+} release may determine speed of muscle shortening and the peak force produced, both of which affect sprint performance (Hirata et al., 2007). We predicted that blocking RyR will decrease whole-animal sprint velocity but increase sustained swimming performance by slowing depletion of Ca^{2+} stores in the SR, which is a known mechanism of muscle fatigue (Stephenson et al., 1998; Allen et al., 2008). (3) Slowing Ca^{2+} re-uptake into the SR by blocking SERCA activity will decrease muscle relaxation rates, as well as reducing force production and fatigue resistance. We tested these hypotheses by conducting a series of experiments in which we used pharmacological manipulation of DHPR, RyR and SERCA to determine their effect on isolated muscle mechanics, swimming kinematics, and maximal sprint and sustained swimming performance in carp, *Cyprinus carpio*.

MATERIALS AND METHODS

Study animals

Carp (*Cyprinus carpio* Linnaeus 1758; mean total length=88.90±1.8 mm; mean mass=18.03±0.91 g) were obtained from A5 Aquatics (Nuneaton, UK, N=40) and Australian Koi Farm (Bringelly, Australia, N=30). All fish were acclimated to 25°C for 1 month before experimentation, and were kept in groups of five to six fish in plastic tanks (645×423×276 mm) that were continuously filtered and aerated. Animals were fed commercial fish food daily, but were starved for 24 h before experimentation. All procedures had the approval of the University of Sydney Animal Ethics Committee (approval number L04/10-2009/2/5158) and were conducted in accordance with the British Home Office Animals (Scientific Procedures) Act 1986, schedule 1.

Swimming performance

We determined sprint velocity by filming the startling response of fish from above (using a Casio Exilim EX F1 camera recording at 60 frames s^{-1}; Shriro Australia Pty Ltd, Sydney, Australia). Fish were introduced into a tank (405×600 mm) filled with water to a depth of 50 mm. When at rest, fish were startled by lightly tapping their tail with a stick. The ensuing escape response was filmed, and the fastest speed recorded in three escape responses was used as the maximum sprint velocity. We analysed videos in Tracker Video Analysis and Modeling Tool (Open Source Physics, www.opensource.physics.org).

Sustained swimming performance was measured as the critical sustained swimming speed (U_{crit}) (Brett, 1964; Hammer, 1995) according to published protocols (Sinclair et al., 2011). We conducted a pilot study (N=6 fish) to determine the time interval between speed increments (t_i=600 s), the speed increment (U_i=0.05 m s^{-1}) and the initial flow rate (0.05 m s^{-1}) so that fish were able to swim for several complete increments before exhaustion (Hammer, 1995). The swimming flume consisted of a 250×50 mm (length×diameter) clear Perspex® tube tightly fitted into the single exit of a Y-shaped rubber connector (total length=150 mm). Into each of the other openings of the Y-connector we inserted an inline submersible pump (Rule iL500 King Pumps, Miami, FL, USA; 12 V), achieving a tight fit. A plastic grid separated the Perspex® flume from the two pumps, and a bundle of hollow straws at the water inlet end of the flume, at the opposite end of the Y-connector, helped maintain laminar flow. The flume and pumps were submerged in a plastic tank (645×423×276 mm). We used a variable DC power source (MP3090, Powertech, Jaycar, Sydney, Australia) to adjust the flow speed, which was calibrated using a flow meter (FP101, Global Water, Gold River, CA, USA).

We determined the sensitivities of sprint and sustained swimming performance to the activities of DHPR and RyR by exposing fish to solutions of nifedipine (Sigma-Aldrich, Castle Hill, NSW, Australia) (Houston 1986) and dantrolene (Sigma-Aldrich) (Van Winkle 1976; Krause et al., 2004), respectively. There are no differences in the principal Ca^{2+} handling mechanisms between fibre types, although they differ in their relative density of DHPR and RyR; therefore, nifedipine and dantrolene will not bind preferentially to one or the other (Antilla et al., 2007; Lee et al., 1991; Berchthold et al., 2000). We estimated initial doses from our previous work on zebrafish (Seebacher and Walter, 2012); these doses were based on published data evaluating the effects of dantrolene and nifedipine in mammals using oral administration [nifedipine, approximately 5–20 μmol l^{-1}; dantrolene, 1–100 μmol l^{-1} (Dykes, 1975; Foster, 1983; Houston, 1986)]. When presented orally, both drugs act within minutes and their half-life is approximately 10–12 and 2–3 h, respectively (Dykes, 1975; Foster, 1983). We first immersed fish in 30 μmol l^{-1} of nifedipine (N=8) or 150 μmol l^{-1} dantrolene (N=8) for 1 h before swimming performance tests. Subsequently, we increased doses to 50 μmol l^{-1} nifedipine and 300 μmol l^{-1} dantrolene. We used the same fish for different drug doses, starting at the lower dose and leaving 24 h between trials, which is sufficient for the effects of the initial doses to wear off (Dykes 1975; Foster et al., 1983). Additionally, we determined swimming performance in a control group (N=8) that was not treated with drugs, and to which we compared the drug treatments.

We determined tail-beat amplitude and frequency as a measure of *in situ* muscle performance during locomotion. We filmed fish (with a Casio Exilim Ex F1 camera filming at 60 frames s^{-1}) swimming steadily at 0.15, 0.25 and 0.35 m s^{-1}, and analysed video in Tracker software. We measured amplitude as the maximal displacement of the tail at the peduncle during one tail-beat cycle. Similarly, we determined period as the time taken for the tail to complete one cycle, and calculated frequency as 1/period. All swimming performance measures were taken at 25°C.

Muscle biomechanics

Fish used to determine biomechanics of isolated muscle were euthanized *via* a blow to the head, transection of the spinal cord and pithing. A bundle of caudal muscle fibres was dissected from each side of the fish (for subsequent muscle mechanics) in cooled (<5°C), oxygenated (95% O_2; 5% CO_2) carp Ringer's solution [composition in mmol l^{-1}: NaCl 115.7, sodium pyruvate 8.4, KCl 2.7, $MgCl_2$ 1.2, $NaHCO_3$ 5.6, NaH_2PO_4 0.64, HEPES sodium salt 3.2, HEPES 0.97, $CaCl_2$ 2.1, pH 7.4 at 20°C (Johnson and Bennett, 1995; Wakeling et al., 2000)]. An aluminium foil T-clip was wrapped round either end of each muscle fibre bundle.

Isometric studies were used to determine the twitch and tetanus kinetics of the isolated caudal muscle fibre bundles. The following methodology was used simultaneously on two Aurora Scientific (Aurora, ON, Canada) setups to compare mechanical performance between the muscle preparations from each side of the fish (one muscle preparation acting as a control, one as drug treatment). The aluminium foil clips were attached at one end of the muscle fibre bundle to a force transducer (400A, Aurora Scientific), and at the other end to a motor arm (322C-I, Aurora Scientific). The muscle preparation was then allowed to equilibrate within the organ bath at 25±0.5°C for 10 min in circulating, oxygenated (95% O_2; 5% CO_2) carp Ringer's solution. The preparation was then held at constant length and subjected to a series of 1.0 ms pulse width stimuli *via* parallel platinum electrodes to generate a series of twitches. Stimulus amplitude (50–70 mA) and muscle length were adjusted to determine the stimulation parameters and muscle length corresponding to maximal isometric twitch force. An isometric tetanic force response was elicited by subjecting the muscle to a 300 ms train of stimulation. Stimulation frequency (110–130 Hz) was altered to determine maximal tetanic force. Time to half peak tetanic force (referred to as tetanus activation time) and time from last stimulus to half tetanic force relaxation (referred to as tetanus relaxation time) were measured *via* AS1600A analysis software (Aurora Scientific). Each tetanus exhibited a distinct peak on the left-hand side followed by a drop in force, then a plateau in force prior to relaxation (Fig. 1). Therefore, peak tetanic force was measured on the left- and right-hand side of each tetanus to monitor whether changes in force, due to fatigue or drug treatment, differentially affected the peak force produced in these different regions of the tetanus. A rest period of 5 min was allowed between each tetanic response. We calculated rates of force production (peak tetanic force/2× time to half peak tetanus) and muscle relaxation (peak tetanic force/2× time from last stimulus to half relaxation) as measures of the contractile performance of muscle.

After the maximal tetanus was achieved, the control Ringer's solution was replaced by either fresh control Ringer's solution (for the control muscle fibre preparation) or 50 μmol l^{-1} dantrolene (Van Winkle, 1976; Fruen et al., 1997), 10 μmol l^{-1} thapsigargin (Kurebayashi and Ogawa, 2001; Galli et al., 2006) or 10 μmol l^{-1} nifedipine Ringer's solution (Weigl et al., 2000) (all drugs purchased from Sigma-Aldrich). Maximal tetanus responses were determined after 10 min of incubation in the drug treatment.

After a further 5 min rest, fatigue resistance was determined by subjecting the muscle preparation to a series of tetani, each of 300 ms stimulation duration, at a rate of one tetanus per second for 200 s. We also determined fatigue in muscle from an additional, independent control group (N=10), to which we compared the drug treatments (see Statistical analysis below).

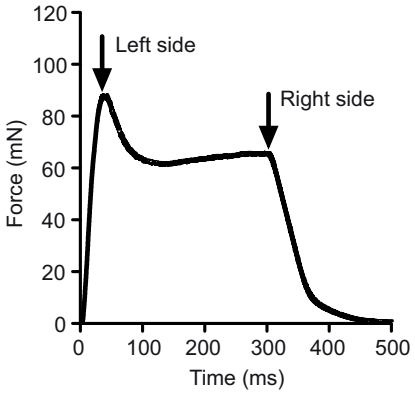

Fig. 1. Example of a typical control tetanus. The muscle was stimulated from 0 to 300 ms. The muscle preparation contains mixed fibre types such that the right peak represents the peak force produced by the slower fibres.

Statistical analysis

Critical sustained swimming speed (U_{crit}) and sprint velocity of fish treated with dantrolene or nifedipine were compared with control fish using *t*-tests. Kinematics data were analysed by ANOVA with treatment as a fixed factor and water flow velocity as a repeated measure; we used Pillai's trace as the test statistic to determine significance for the repeated measure. We performed separate analyses for the dantrolene and nifedipine treatments. Of the nifedipine-treated fish, only four reached swimming velocities of 0.25 m s^{-1}, and only two reached 0.35 m s^{-1}. Hence, we could not analyse the effect of nifedipine of swimming kinematics at 0.35 m s^{-1}.

Muscle mechanics data were analysed with paired *t*-tests, comparing performance of the untreated control muscle with that of the drug-treated muscle. We performed separate analyses for dantrolene, thapsigargin and nifedipine treatments. We analysed muscle fatigue by comparing each treatment with the control at tetanus numbers 10, 50, 100 and 200. Data were expressed as percent of the force produced in the first tetanus. We used PERMANOVA (in Primer 6 software, Primer-E, Plymouth, UK) with treatment (control and each drug treatment) as a fixed factor and tetanus number as a random factor. The truncated product method (Zaykin et al., 2002) was used to combine all the *P*-values in this study to determine whether there is a bias from multiple hypothesis testing. The truncated product method *P*-value was <0.0001, showing that the results are not biased.

RESULTS

Swimming performance

Neither U_{crit} nor sprint performance changed with exposure to 150 μmol l^{-1} dantrolene (t=1.59, P=0.13 and t=0.25, P=0.81, respectively). A dantrolene concentration of 300 μmol l^{-1} did not affect U_{crit} (t=1.33, P=0.20), but it significantly decreased sprint velocity (t=2.28, P<0.05; Fig. 2). Exposure to 30 μmol l^{-1} nifedipine did not affect swimming performance (U_{crit}: t=1.32, P=0.21; sprint velocity: t=1.68, P=0.12), but 50 μmol l^{-1} nifedipine significantly reduced U_{crit} (t=2.79, P<0.02) and sprint velocity (t=4.99, P<0.0001; Fig. 2).

Swimming kinematics

Tail-beat amplitude increased significantly with increasing water flow ($F_{2,10}$=5.52, P<0.03; Fig. 3A), and dantrolene did not affect

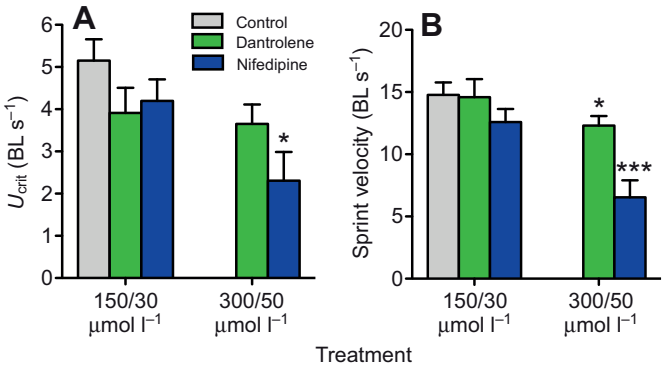

Fig. 2. (A) Critical sustained swimming speed (U_{crit}) and (B) sprint velocities in control (untreated, grey bars), dantrolene- (green bars) and nifedipine-treated (blue bars) carp. Fish were exposed to two doses of each drug (150 µmol l^{-1} dantrolene and 30 µmol l^{-1} nifedipine, and 300 µmol l^{-1} dantrolene and 50 µmol l^{-1} nifedipine), and the higher dose of nifedipine significantly reduced U_{crit}, whereas the higher doses of each drug significantly reduced sprint velocities (*P<0.05; ***P<0.001). BL, body lengths.

tail-beat amplitude compared with control fish ($F_{1,11}$=1.98, P=0.19). Tail-beat frequency increased with increasing flow ($F_{2,10}$=25.57, P<0.0001), but the increase was significantly less in dantrolene-treated fish compared with controls (interaction between treatment and flow velocity, $F_{2,10}$=4.75, P<0.05; Fig. 3B).

Similar to the dantrolene treatment, amplitude did not change as a result of the nifedipine treatment ($F_{1,9}$=0.012, P=0.92; Fig. 3A), but there was a significant interaction between flow speed and frequency as a result of nifedipine treatment ($F_{1,9}$=6.49, P<0.05; Fig. 3B).

Effect of drug treatments on muscle biomechanics

Exposure to dantrolene significantly reduced tetanus relaxation times, and also decreased peak tetanus force (Fig. 4, left column; Table 1). However, dantrolene did not affect activation time, or the rates of muscle activation or relaxation (Figs 4, 5, Table 1). Thapsigargin did not significantly affect tetanus activation or relaxation times, or peak tetanus force (Fig. 4, right column; Table 1). Thapsigargin did, however, significantly increase the rates of force production and muscle relaxation (Fig. 5, Table 1). Interestingly, nifedipine increased

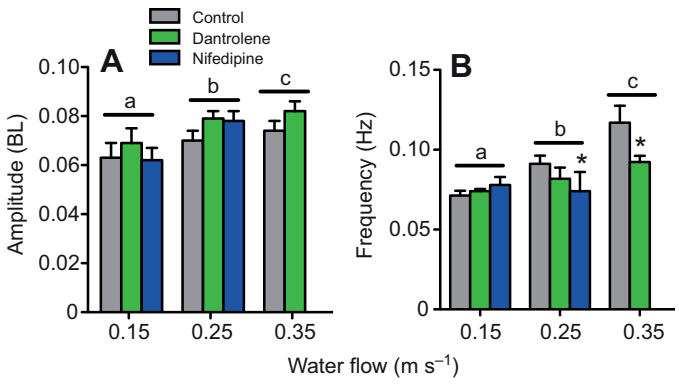

Fig. 3. (A) Tail-beat amplitudes and (B) frequencies during steady swimming in carp at different water flow speeds. Data for control (grey bars), dantrolene- (green bars) and nifedipine-treated (blue bars) fish are shown. Significant differences between drug-treated and control fish are indicated by asterisks (*P<0.05), and differences between flow speeds are indicated by different letters placed above horizontal bars.

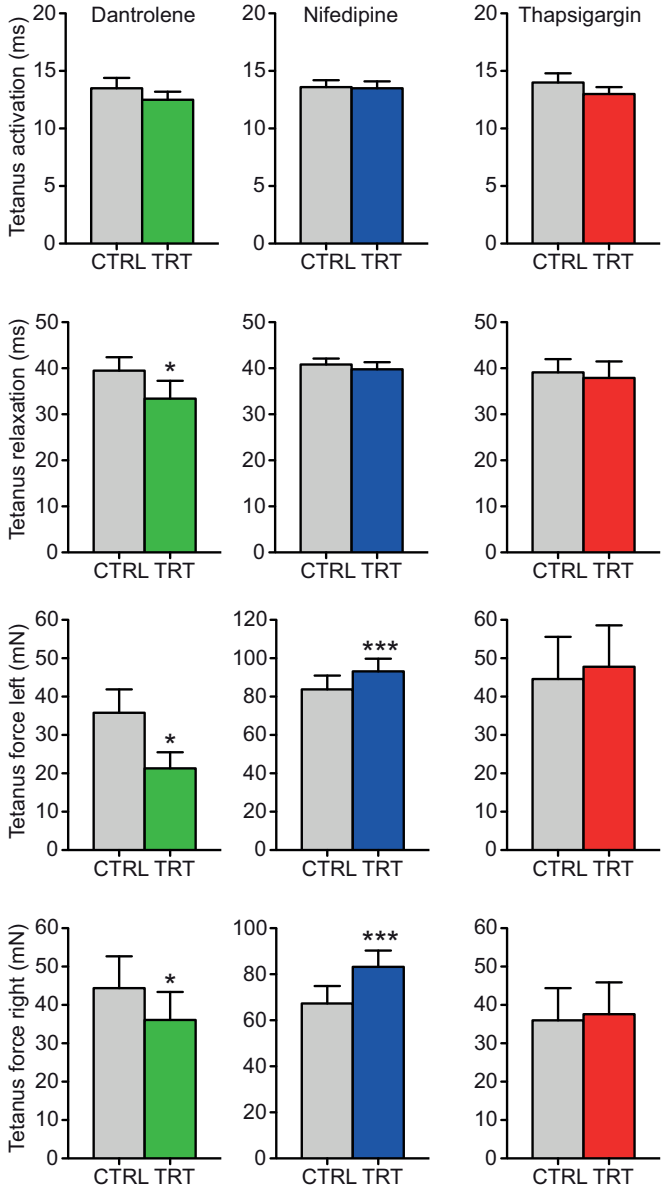

Fig. 4. Effect of the ryanodine receptor blocker dantrolene (left column), the dihydropyridine receptor blocker nifedipine (centre column) and the SERCA blocker thapsigargin (right column) on tetanus mechanics of carp tail muscle bundles. Asterisks indicate significant differences between drug-treated muscle (TRT) and control muscle (CTRL; grey bars) (*P<0.05; ***P<0.001).

peak tetanic force, but had no effect on muscle activation and relaxation times (Fig. 4, centre column; Table 1), so that rates of force production and relaxation also increased significantly following nifedipine treatment (Fig. 5, Table 1).

During the fatigue runs, tetanus force of the left- and right-hand sides decreased with increasing tetanus number in all treatments (all pseudo $F_{3,72}$>12.0, P<0.001; Fig. 6). Dantrolene had no effect on the left-hand side of the tetanus (pseudo $F_{1,72}$=0.30, P=0.78; Fig. 6A) but significantly attenuated the decline in force production (i.e. it increased fatigue resistance) of the right-hand side compared with controls (pseudo $F_{1,72}$=15.63, P<0.02; Fig. 6B). Thapsigargin did not affect force production of the left-hand side of the tetanus compared with controls (pseudo $F_{1,72}$=3.93, P=0.12; Fig. 6A), but

Table 1. Effects of the RyR blocker dantrolene, the DHPR blocker nifedipine and the SERCA blocker thapsigargin on tetanus biomechanics in the carp *Cryprinus carpio*

	Dantrolene		Nifedipine		Thapsigargin	
	t	*P*	*t*	*P*	*t*	*P*
Peak tetanic force, left (mN)	2.63	0.027	–8.42	<0.0001	–1.85	0.098
Peak tetanic force, right (mN)	3.14	0.012	–9.92	<0.0001	–1.34	0.21
Activation time (ms)	2.26	0.050	0.41	0.69	1.65	0.13
Activation rate ($N\,s^{-1}$)	2.13	0.062	–5.00	<0.001	–3.58	0.006
Relaxation time (ms)	3.17	0.011	1.15	0.28	1.16	0.28
Relaxation rate ($N\,s^{-1}$)	–0.51	0.62	–4.93	<0.001	–3.56	0.006

Results of paired *t*-tests comparing drug treatments with the control are shown.
DHPR, dihydropyridine receptor; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca^{2+}-ATPase.

force production declined more rapidly (i.e. fatigue resistance was decreased) on the right-hand side following thapsigargin treatment (pseudo $F_{1,72}$=4.91, P<0.05; Fig. 6B). Nifedipine caused a significantly faster decline in force production with increasing tetanus number (i.e. decreased fatigue resistance) in both the left- and right-hand sides compared with controls (left: interaction pseudo $F_{3,72}$=9.38, P<0.001; right: interaction pseudo $F_{3,72}$=8.21, P<0.001; Fig. 6).

DISCUSSION

High force production and rapid rates of muscle activation and relaxation are linked mechanistically to high sprint performance (Spierts and Van Leeuwen, 1999). As we predicted, the decrease in isolated peak muscle force following RyR blockade was paralleled by a decrease in whole-animal sprint performance. The decrease in force can be explained by reduced Ca^{2+} release from the SR, decreasing the number of actinomyosin cross-bridges (Berchtold et al., 2000). However, reduced RyR activity following dantrolene treatment did not decrease muscle activation times. Unexpectedly, dantrolene reduced muscle relaxation times (Table 2). The latter may reflect faster re-sequestration of Ca^{2+} into the SR as a result of the lower concentration of Ca^{2+} associated with reduced RyR activity and force production. Accordingly, rates of muscle relaxation remained unaffected by dantrolene. In contrast, rates of force production and relaxation decreased significantly in rat muscle following dantrolene treatment (James et al., 2011). The increase in fatigue resistance following dantrolene treatment may reflect the reduced depletion of Ca^{2+} stores in the SR associated with lower RyR activity (Stephenson et al., 1998); note that dantrolene treatment does not block RyR completely, so there will be residual Ca^{2+} release from the SR (van Winkle, 1976). However, this increase in fatigue resistance of isolated muscle did not translate to increased sustained swimming performance. U_{crit} and burst swimming velocities of our control fish were similar to those reported in the literature (Heap and Goldspinck, 1986; Wakeling et al., 2000). Carp increased swimming speed by concomitant increases in tail-beat amplitude and frequency. The reduction in tail-beat frequency at higher swimming speed when RyRs were blocked indicates that the whole muscle became slower during swimming and/or the reduction in muscle force production during dantrolene treatment compromised the fish's ability to overcome the greater drag forces involved at higher tail-beat frequencies. This observation is not reflected in the biomechanics of a single tetanus. There was an indication that activation rates of the tetanus decreased when RyRs were blocked, but this was not significant (P=0.062). However, this reduction in activation rates may become more pronounced with repeated tetanus stimulation, and muscle activation time and swimming speed in fish are often correlated (Wardle, 1975). RyRs can affect muscle in several ways and, as outlined above, increased activity increases force production but at the same time depletes Ca^{2+} stores in the SR, thereby inducing fatigue. Hence, at the same time, blocking RyRs may be detrimental by reducing force production and beneficial by reducing SR Ca^{2+} depletion, so that there is no net effect on U_{crit}. This is in contrast to sprint performance, where SR Ca^{2+} depletion would be relatively unimportant, so that blocking RyR would have a detrimental effect only, as observed.

DHPRs mediate excitation–contraction coupling and release Ca^{2+} into the myocyte upon nerve stimulation. In heart muscle, DHPRs stimulate RyRs by Ca^{2+} release into the myocyte (Shiels et al., 1999; Tiitu and Vornanen, 2003). In contrast, activation of RyR by DHPR in skeletal muscle is achieved by mechanical coupling between the two receptors, which is independent from the Ca^{2+} release (Dirksen, 2009). Nifedipine blocks the Ca^{2+} current across the sarcolemma but does not inhibit the direct interaction between DHPR and RyR (Fleckenstein, 1983). Hence, in isolated muscle there is no negative effect of nifedipine on force production. On the contrary, nifedipine increased force production, but also decreased fatigue resistance of isolated muscle. These responses were consistent across all animals but are not easily explained. It may be

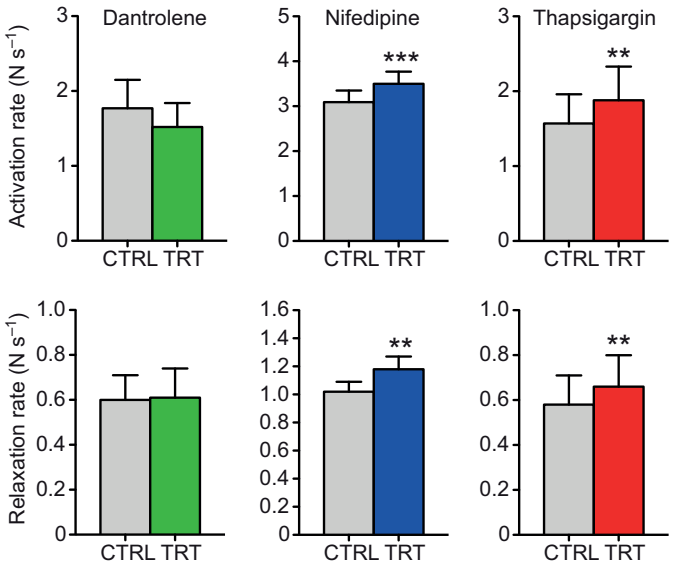

Fig. 5. Effect of dantrolene, nifedipine and thapsigargin on rates ($N\,s^{-1}$) of muscle activation and relaxation in carp. Asterisks indicate significant differences between drug-treated muscle (TRT) and control muscle (CTRL; grey bars) (**P<0.01; ***P<0.001).

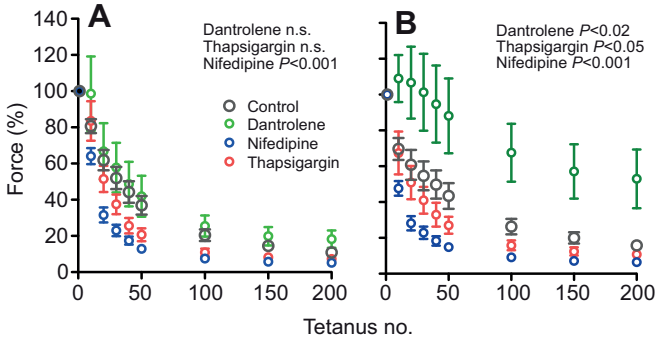

Fig. 6. Fatigue (force produced as a percentage of first tetanus) over 200 consecutive tetani for control, dantrolene, thapsigargin and nifedipine treatments. Force produced on the left- (A) and right-hand sides (B) of the tetanus are shown separately. Significant differences from control values are indicated in each panel.

Table 2. Summary of the effects of blocking dihydropyridine receptors (DHPRs; nifedipine), ryanodine receptors (RyRs; dantrolene) and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA; thapsigargin) on swimming performance, swimming kinematics and isolated muscle mechanics in the carp *Cyprinus carpio*

	Dantrolene	Nifedipine	Thapsigargin
Locomotion			
Sprint	–	–	n/a
Sustained	No effect	–	n/a
Kinematics			
Amplitude	No effect	No effect	n/a
Frequency	–	–	n/a
Isolated muscle			
Peak tetanic force	–	+	No effect
Activation time	No effect	No effect	No effect
Activation rate	No effect	+	+
Relaxation time	–	No effect	–
Relaxation rate	No effect	+	+
Fatigue resistance	+	–	–

+, Increase in the response variable; –, decrease in the response variable. No effect, no change after pharmacological blockade.

that the Ca^{2+} released by DHPR has an inhibitory effect on RyR at the same time as the molecular interaction stimulates RyR, thereby maintaining a 'tone' on RyR activity. The activity of RyR is regulated by Ca^{2+} concentrations, whereby the receptor is activated by low [Ca^{2+}] (0.5 µmol l^{-1}) and inhibited by higher [Ca^{2+}] (0.15 mmol l^{-1}) (Berchtold et al., 2000). This scenario would explain the data from our isolated muscle experiments, but is highly speculative and must be confirmed experimentally.

Reduced fatigue resistance of isolated muscle following nifedipine treatment is paralleled by decreases in tail-beat frequency and U_{crit}. These findings indicate a direct effect of muscle mechanics on whole-animal performance. The decrease in U_{crit} may have been further enhanced by the depressing effect of DHPR inhibition on the cardiovascular system (Fleckenstein, 1983; Vornanen, 1997; Galli et al., 2006). However, sprint performance also decreased with DHPR inhibition, which may indicate a role of the cardiovascular system in facilitating burst activity, which is unlikely, or a role in muscle contraction of the Ca^{2+} released by DHPR into the myocyte. The fact that concentrations of DHPR increase with endurance training (Antilla et al., 2006), and that sprint performance decreases with a decrease in DHPR concentration (Hirata et al., 2007) confirms that there is a positive relationship between the density of this receptor and whole-animal locomotion. Undoubtedly, this role is in large part mediated by the direct interaction between DHPR and RyR, but our data indicate that there is also a more complex functional relationship that does not involve this direct interaction (i.e. as indicated by the effect of nifedipine). In addition to its role as a rapid-acting voltage sensor during excitation–contraction coupling, the DHPR is also a slow-acting voltage-gated L-type Ca^{2+} channel (Dirksen, 2009). This slow mode of action may be important in replenishing Ca^{2+} stores (Nakai et al., 1996; Bannister et al., 2009; Dirksen, 2009), which is essential to increase fatigue resistance (Kurebayashi and Ogawa, 2001; Pan et al., 2002). The fact that tail-beat frequency decreased and few fish continued swimming at moderate (compared with control) water flow velocities is consistent with fatigue induced by depletion of Ca^{2+} stores.

The moderate, but significant, increases in activation and relaxation rates of isolated muscle following thapsigargin treatment are unexpected. For a single tetanus, it may point towards increased reliance on alternative and faster Ca^{2+} sequestration mechanisms, such as parvalbumin (Arif, 2008), when SERCA activity is low. Single tetanus force production of isolated muscle was not affected by thapsigargin treatment, which indicates that Ca^{2+} stores in the sarcoplasmic reticulum are not depleted during a single tetanus to the extent that reduced SERCA activity, and thus slower replenishing of Ca^{2+} stores, would inhibit force production (Allen et al., 2008). Again, re-sequestration of Ca^{2+} from the cytosol by parvalbumin would facilitate muscle relaxation at least in the short term, and may thereby help maintain force production and swimming performance (Seebacher and Walter, 2012). Isolated mouse soleus muscle in which SERCA2 gene expression was disrupted also showed no decrease in force production, although there was an increase in relaxation times (Sjåland et al., 2011), which may indicate that other SERCA isoforms partially compensated for the reduction in SERCA2 activity, and that store depletion did not reduce force production over the time frame of measurement. In the longer term, however, thapsigargin treatment decreased fatigue resistance of carp muscle, suggesting that ultimately SERCA activity is essential in re-uptake of Ca^{2+} into the SR to avoid store depletion and fatigue. Again, there are differences between our findings from carp here and our previous work on rat muscle, where thapsigargin did not affect rates of force production or muscle relaxation, although as in carp it did reduce fatigue resistance (James et al., 2011).

We have shown that different molecular mechanisms involved in Ca^{2+} cycling during excitation–contraction coupling affect the same whole-animal traits, and that the same molecular trait, such as RyR activity, affects whole-animal locomotion differently in different species. The strength of uncovering the mechanistic basis of whole-animal function lies in providing explanatory models for observed variation within and between species. Hence, questions addressing the evolutionary processes that lead to differences between species or populations should focus on underlying mechanisms rather than solely on the whole-organism trait.

FUNDING

This research was in part supported by an Australian Research Council grant to F.S.

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