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RESEARCH ARTICLE

Differences in locomotor performance between individuals: importance of parvalbumin, calcium handling and metabolism

Frank Seebacher[1,*] and Isabel Walter[1,2]

[1]School of Biological Sciences A08, University of Sydney, NSW 2006, Australia and [2]Energy Metabolism, German Institute of Human Nutrition, Potsdam-Rehbrücke, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Germany

*Author for correspondence (frank.seebacher@sydney.edu.au)

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SUMMARY

Locomotor performance is linked to fitness and health of animals and is expected to be under strong selection. However, interindividual variation in locomotor performance is pronounced in many species. It was our aim to investigate the relative importance of energy metabolism and calcium handling in determining sprint and sustained locomotion in the zebrafish (*Danio rerio*). Sprint and sustained performance (U_{crit}) varied independently from each other. Using *in vivo* electroporation, we found that increased parvalbumin protein concentration improved both sprint and sustained locomotion. This is the first demonstration that parvalbumin plays a role in determining whole-animal performance. High sprint performance fish had greater mRNA concentrations of the metabolic regulators PPARδ and PGC1β compared with fish with poor sprint performance. High sustained performance fish, in contrast, had greater concentrations of PGC-1α and PGC-1β. The increased expression of these metabolic regulators indicates an enhancement of the metabolic machinery in high performance animals. Sprint performance is also enhanced by creatine kinase activity, which may be associated with increased PPARδ mRNA concentration. Ryanodine receptor (RyR) and sarcoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA1) mRNA concentrations were significantly increased in high sustained performance fish, while parvalbumin 2, dihydropyridine (DHPR) receptor and SERCA2 mRNA levels were increased in fish with high sprint velocities. Sustained performance was more sensitive to experimentally induced decreases in RyR and DHPR activity than sprint performance. We provide mechanistic explanations of why locomotor performance differs between individuals, which is important for understanding ecological and sporting success, disease and the evolutionary processes underlying selection.

Key words: sustained locomotion, sprint performance, ryanodine receptor, SERCA, dihydropyridine receptor, gene expression, electroporation.

INTRODUCTION

Locomotor performance is closely linked to fitness because it facilitates behaviour including prey capture, predator escape, aggression and reproduction (Clobert et al., 2000; Husak et al., 2006; Wilson et al., 2010). Interindividual variation in locomotor performance is pronounced in many species (Irschick and Losos, 1998; Van Damme et al., 2002; LeGalliard et al., 2004). This variation is interesting at an evolutionary level, because although selection acts on whole-organism phenotypes such as locomotion or metabolic rate, responses to selection and the evolutionary process itself occurs at the level of genes and their expression (Lande and Arnold, 1983). Complex functions such as locomotion are likely to depend on numerous genes and the function of their protein products (Arnold, 1983; Bauwens et al., 1995). Independent evolution of these underlying traits, and trade-offs with other fitness-related functions (Clobert et al., 2000), may cause variation in locomotor performance between individuals. Success may therefore ultimately depend on the suite of underlying genetic and biochemical traits. Hence, understanding the underlying mechanisms will advance knowledge from a correlation between locomotion and an ecological or behavioural variable such as fighting success to a functional understanding of the enabling mechanisms that lead to success (Sinclair et al., 2011). Conversely, understanding the mechanistic basis of locomotion will provide insight into pathological conditions (Ingham, 2009). Hence, it was our aim to investigate the importance of muscle energy metabolism and calcium handling in determining differences in sprint and sustained locomotion between individual zebrafish (*Danio rerio*).

Animal morphology directly affects the biomechanics of locomotion (Webb and Weihs, 1983; Lauder, 2005; Langerhans and Reznick, 2008). Within species, however, differences in the morphology of the skeletal system pose only a limited constraint on locomotion (Vanhooydonck et al., 2001). Muscle contractile properties are therefore likely to be the principal mechanisms determining differences in locomotor performance between individuals of the same species (James et al., 1995). Apart from the composition of the contractile apparatus itself, such as myosin isoforms and their activity (Johnston and Walesby, 1977; Johnston and Temple, 2002), the principal biochemical mechanisms that determine force and rates of muscle contraction and relaxation are energy metabolism (Garland and Else, 1987; Cano and Nicieza, 2006; Joyner and Coyle, 2008) and calcium handling (Berchtold et al., 2000; Antilla et al., 2008). The former provides the energy (adenosine triphosphate, ATP) necessary for muscle contraction and relaxation, and the latter mediates muscle excitation–contraction–relaxation coupling and determines contraction and relaxation rates.

Nerve signals are coupled to muscle contraction by stimulating the voltage gated calcium channels dihydropyridine receptors (DHPR)

(Berchtold et al., 2000). DHPR release calcium (Ca^{2+}) into the cell, and stimulate ryanodine receptors (RyR) to release additional Ca^{2+} from stores in the sarcoplasmic reticulum (Dirkson, 2009). The resultant Ca^{2+} signal acts on troponin and causes actin and myosin to interact, thereby leading to muscle contraction (Farah and Reinach, 1995). Muscle relaxation is facilitated by the activity of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} back into the sarcoplasmic reticulum (Berchtold et al., 2000). The rate of Ca^{2+} resequestration, and thereby muscle relaxation, is enhanced by parvalbumin, which initially binds Ca^{2+} before it is transported into the sarcoplasmic reticulum by SERCA (Arif, 2008).

During this Ca^{2+} mediated cycle of contraction and relaxation, ATP is consumed by the interaction between myosin and actin, and by SERCA pumping. Hence, the different steps of Ca^{2+} release and resequestration, as well as the availability of ATP may constrain muscle function and locomotion. However, the relative importance of these mechanisms for whole-animal locomotor performance and its variation between individuals is unresolved (Gibb and Dickson, 2002; James et al., 2005; Plomgaard et al., 2006; James et al., 2011). Additionally, sprint and endurance performance are likely to depend on different underlying mechanisms, and there may be a trade-off between the two so that maximising one will cause a decrease in the other (Van Damme et al., 2002; Wilson and James, 2004; James et al., 2011).

We tested the hypotheses that (i) there is a trade-off between sprint and sustained locomotor performance and that (ii) sprint and sustained performance are facilitated by increased expression of Ca^{2+} handling proteins (DHPR, RyR, SERCA1 and 2), and of transcriptional regulators of metabolism (PPARδ, PGC-1α, PGC-1β) (Scarpulla, 2008). At a protein level, we predicted that (iii) sprint performance is associated with increased activity of the fast acting ATP-release enzyme creatine kinase (Saks, 2008) and the glycolytic enzyme lactate dehydrogenase, and that sustained performance is linked to greater activity of the mitochondrial enzyme citrate synthase (Seebacher and Glanville, 2010). With respect to Ca^{2+} handling proteins, we predicted (iv) that inhibition of DHPR will affect sustained performance negatively, while inhibition of RyR will decrease sprint performance (Hirata et al., 2007; James et al., 2011). Lastly, we tested the hypothesis that (v) increased parvalbumin expression will enhance sprint and sustained locomotion (Arif, 2008; James et al., 2011).

MATERIALS AND METHODS

Animal husbandry

Adult short fin zebrafish, *D. rerio* (F. Hamilton 1822), were obtained from commercial suppliers. Eight to 10 fish were kept per holding tank (390×260×195 mm) and were fed *ad libitum* with fish flakes (Hartz Mountain Corporation, Secaucus, NJ, USA). Tanks were under a 12 h light:12 h dark regime, and water temperature was kept at 25±1°C during the holding period and all experiments. Holding tanks contained gravel and plastic plants, and were continuously filtered. All experiments were carried out with the approval of the University of Sydney Animal Experimentation Ethics Committee (approval number L04/1-2010/2/5189).

All fish were kept in holding tanks for 1 week before the beginning of the experiments. Total length (mouth to peduncle) and body mass of each fish were measured before determination of swimming performance. On average, fish weighed 4.27±0.026 g (mean ± s.e.m.), and mean ± s.e.m. length was 2.90±0.061 cm. From these measurements we calculated a condition factor k=(mass×100)/length^3, and we used only fish with condition factors between 1.4 and 2.1 in experiments.

Swimming performance

We determined sprint velocity and critical sustained swimming performance of N=46 fish. Sprint velocity was measured by filming the startling response of fish from above (using a Casio Exilim EX F1 camera recording at 30 frames s^{-1}). Fish were introduced into a tank (405×600 mm) filled with water to a depth of 25 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 software (Open Source Physics, www.opensourcephysics.org).

Sustained swimming performance was measured as the critical sustained swimming speed (U_{crit}) (Brett, 1964; Sinclair et al., 2011). We conducted a pilot study (N=10 fish) to determine the time interval between speed increments (t_i=600 s), the speed increment (U_i=0.06 m) and the initial flow rate (0.2 m). The swimming flume consisted of a 150 mm length × 39 mm diameter clear Perspex tube tightly fitted into the single exit of a Y-shaped rubber connector (total length, 0.15 m). Into each of the other openings of the Y-connector we inserted an inline submersible pump (12 V, iL500, Rule, Miami, FL, USA), 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, Osborne Park, WA, Australia) to adjust the flow speed, which was calibrated using a flow meter (FP101, Global Water, Gold River, CA, USA).

After measurement of swimming performance, fish were killed with an overdose of buffered MS222 (0.3 g l^{-1}, pH 7.0; Sigma, Castle Hill, NSW, Australia). The tail was skinned, and the total tail muscle was removed from each side of the tail; one lateral portion was placed into RNAlater (Applied Biosystems, Sydney, Australia) for gene expression analysis, the other immediately frozen in liquid nitrogen for enzyme activity assays.

Pharmacological treatments

To determine the sensitivity of sprint and sustained locomotion to the activity of DHPR and RyR, we exposed fish to solutions of nifedipine (Sigma) and dantrolene (Sigma), respectively. To the best of our knowledge, there are no published experiments using these treatments on live fish. Hence, we estimated initial doses from cell culture studies (van Winkle, 1974; Weigl et al., 2000) with the aim of increasing concentrations until an effect was observed in either sprint or sustained locomotion. We immersed fish in 20 μmol l^{-1} nifedipine (N=12) and 50 μmol l^{-1} dantrolene (N=12) for 1 h. Dantrolene did not affect either sprint or sustained performance at that concentration so we conducted a further treatment of exposure to 150 μmol l^{-1} dantrolene for 1 h (N=12). All fish recovered well from the drug treatments.

In vivo electroporation

To test whether parvalbumin protein concentration affects whole-animal locomotor performance, we overexpressed parvalbumin 4 (parv4; GenBank NM_212783.2) in tail muscle by *in vivo* electroporation (Rambabu et al., 2005). We conducted two treatments, injecting a plasmid (pEGFP-N1; Clontech, Mountain View, CA, USA) containing green fluorescent protein (GFP) only to control for the electroporation procedure, and a plasmid containing both our parv4 target gene plus GFP. In the latter, parv4 and GFP are transcribed and translated with the same efficiency. When

expressed, GFP protein is detectable under a fluorescence microscope so inclusion of the GFP gene allowed us to screen fish and select only those animals in which both GFP and parv4 proteins were expressed across the whole tail muscle (see below).

Vector construction, generation and purification were conducted by GenScript (GenScript Co., Piscataway, NJ, USA). Briefly, the nucleotide sequence of parv4 (see above) was integrated into the pUC57 vector, and subcloned into the pEGFP-N1 plasmid (Cloning site: *Xho*I–*Bam*HI). Plasmids were diluted in sterile 0.9% saline. Fish were anaesthetised with buffered MS222 (0.1 g l^{-1}, pH 7.0; Sigma), and we injected the plasmid solution into the tail muscle along the length of the tail (2–3 injections) at a concentration of 30 μg of plasmid in 20 μl saline using an insulin syringe (31 gauge). Injection was immediately followed by 6 pulses of 25 V applied across the tail muscle *via* a pair of tweezer electrodes connected to an Isolated Pulse Stimulator (Model 2100, A-M Systems, Sequim, WA, USA). After electroporation, fish were allowed to recover from the anaesthetic (5–10 min) before being returned to their home tank. Six days after electroporation the fish were anaesthetised as described above to assess GFP (and, hence, parv4 in the experimental animals) protein expression under a fluorescence microscope (Zeiss, Oberkochen, Germany). We selected only those fish in which GFP and parv4 protein expression covered the whole tail muscle (N=13 for GFP only, and N=27 for parv4+GFP) to measure swimming performance the next day (i.e. 24 h after anaesthesia). As a further test that our electroporation procedure was effective, we collected muscle tissue samples after measuring swimming performance to determine parv4 mRNA expression in N=8 fish from each treatment by qRT-PCR. Parv4 mRNA concentration was significantly higher (×1.98 relative expression) in electroporated fish with the GFP+parv4 insert compared with fish with only the GFP vector (t=2.74, P<0.02; see below for details of analysis).

mRNA concentration

RNA was extracted from 50 mg of tail muscle samples using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA), following the manufacturer's instructions. RNA quality and concentration were verified using a Bioanalyzer (Agilent Biotechnologies, Palo Alto, CA, USA). A 1 μg sample of total RNA was treated with DNAse I (Sigma) and reverse-transcribed using RNAse H-MMLV reverse transcriptase (Bioscript, Bioline, London, UK) and random hexamer primers (Bioline). Quantitative RT-PCR was performed on an Applied Biosystems 7500 qRT-PCR machine (Applied Biosystems, Scoresby, VIC, Australia) according to published protocols (Walter and Seebacher, 2007).

Pre-validated TaqMan® Gene Expression Assays (Applied Biosystems) were used according to the manufacturer's instructions to quantify parvalbumin 2 (pvalb2; assay ID: Dr03432931_g1), parvalbumin 4 (pvalb 4; Dr03095005_m1), Ca^{2+}-transporting-ATPase 1 (atp2a1; Dr03102406_m1), Ca^{2+}-transporting-ATPase 2b (atp2a2b; Dr03097145_m1), dihydropyridine receptor (cacna1s; Dr03173368_sH) and ryanodine receptor 1b (ryr1b; Dr0307391_m1) expression using Taqman Gene Expression Mastermix (Applied Biosystems) with the standard PCR protocol as recommended by the manufacturer. All other primers were designed from sequences obtained from GenBank: AY998087 for peroxisome proliferator activated receptor γ coactivator-1α (PGC-1α), NW_001511055 for peroxisome proliferator activated receptor coactivator-1β (PGC-1β), AF342937 and NM_131468 for peroxisome proliferator activated receptor δa and b (PPARδa and b), respectively (consensus sequence), and AF398343.1 for ribosomal 28S rRNA, which was used as a housekeeping gene. Real-time PCR reactions contained 1× SensiMixPlus SYBR (Quantace, London, UK), 4.5 mmol l^{-1} $MgCl_2$, 50–900 nmol l^{-1} primer and ~50 ng cDNA. The cycle consisted of 95°C for 7 min, 40 cycles of 95°C for 20 s, 60°C for 1 min. Dissociation curve analysis was performed after the amplification step to verify the presence of only a single PCR product.

Enzyme assays

Maximal activity of regulatory enzymes in metabolic pathways determines the maximum flux through that pathway, and may thereby limit ATP production and locomotion. Hence, enzyme activity is a useful measure to assess whether metabolic capacity is limiting (Seebacher et al., 2003). We measured the activity of creatine kinase and lactate dehydrogenase to assess the capacity of rapid, anaerobic ATP production, and the activity of citrate synthase to determine mitochondrial capacity. Tail muscle was collected from the 46 fish for which we determined swimming performance immediately after they had been killed, transferred into liquid nitrogen and stored at –80°C for later analysis. Enzyme activity was determined according to published protocols (Garenc et al., 1998; Seebacher et al., 2003).

Statistical methods

We used regression analysis to test whether variation in sprint performance was related to U_{crit}. We compared enzyme activity between high and low performing fish of the 46 fish we swam initially by selecting the 15 fish with the greatest U_{crit} or sprint performance and comparing these with the 15 fish with the lowest performance. Comparisons were made by t-tests for independent samples, and sprint velocity and U_{crit} were analysed separately. We analysed mRNA concentration of the high and low performing fish by determining expression in high performing fish relative to low performing fish according to Pfaffl (Pfaffl, 2001) and normalising target gene data with 28S expression. We used one-sample t-tests to determine whether relative expression of target genes differed significantly from 1. Similarly, we analysed parv4 expression in electroporated fish by calculating expression of parv4+GFP fish relative to GFP-only fish and testing for divergence from unity with one-sample t-tests.

We compared (with t-test for independent samples) swimming performance of drug treated fish with that of a control (N=12 fish) drawn randomly from our initial group of 46 fish; the random control sample had the same mean as the total sample of 46 fish, albeit with greater variation reflecting the smaller sample size (sprint velocity: control, 20.06±1.58 BL s^{-1}; total sample, 20.01±0.75 BL s^{-1}; U_{crit}: control, 13.71±1.21 BL s^{-1}; total sample, 13.71±0.67 BL s^{-1}). Swimming performance of fish in which parv4 was overexpressed (parv4+GFP) was compared with that of fish in which only GFP was overexpressed by t-tests for independent samples. All statistical analyses were performed in SPSS 18, and data are presented as means ± s.e.m. All P-values are two-tailed except where stated and when our hypotheses made specific predictions about the direction of differences between experimental groups. 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 <0.001, showing that the results are not biased.

RESULTS

There was no relationship between sprint performance and U_{crit} ($F_{1,44}$=0.62, P=0.43; Fig. 1A). The top 15 performers had significantly faster sprint speed (t=15.15, P<0.0001) and U_{crit} (t=17.32, P<0.0001) compared with the slowest 15 fish (Fig. 1B).

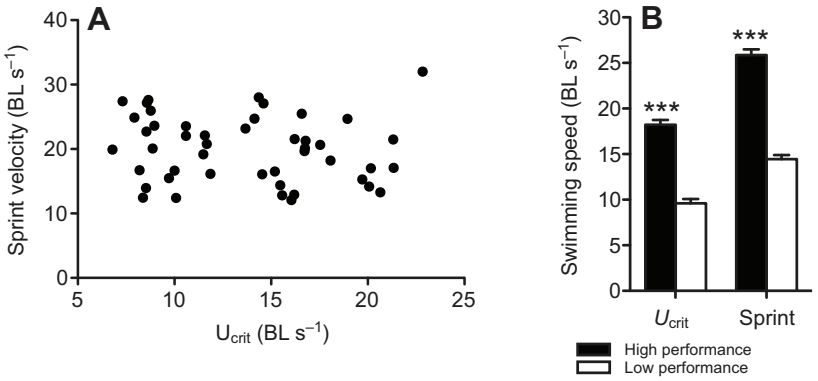

Fig. 1. Sprint swimming velocity of zebrafish varied independently from critical sustained swimming speed (U_{crit}; A). In the analysis we compared fish with the highest sprint velocity and U_{crit} (N=15 each; high performance) with those with the lowest swim performance (N=15 each, low performance; B). BL, body lengths. Significant differences are indicated by asterisks (***P<0.001).

The high sprint velocity group had significantly greater mRNA concentrations of parvalbumin 2 (parv2, t=2.33, P<0.05), DHPR (t=2.56, P<0.03), SERCA 2 (t=1.96, one-tailed P<0.05), PPARδ (t=1.89, one-tailed P<0.05) and PGC-1β (t=1.88, one-tailed P<0.05) compared with the low sprint velocity group; there were no differences between groups in the expression of any of the other genes (all t<1.16, P>0.15; Fig. 2).

Compared with the low U_{crit} group, fish in the high U_{crit} group had significantly greater mRNA concentrations of SERCA1 (t=2.29, P<0.05), RyR (t=3.86, P<0.01), PGC-1β (t=3.39, P<0.01) and PGC-1α (t=2.41, P<0.05); there were no differences between groups in the expression of any of the other genes (all t<1.60, P>0.15; Fig. 2).

Creatine kinase activity of the high sprint velocity fish was significantly greater than that of the low velocity group (t=2.10, P<0.05; Fig. 3), but there were no differences between groups in LDH (t=0.25, P=0.81) or CS (t=1.49, P=0.15) activities. Similarly, none of the enzyme activities differed between the high and low U_{crit} groups (all t<1.15, P>0.25; Fig. 3).

Exposure to 20 μmol l^{-1} nifedipine significantly reduced U_{crit} compared with controls (t=2.64, P<0.02), but did not alter sprint velocity (t=0.81, P=0.43; Fig. 4A). Dantrolene at 50 μmol l^{-1} had no effect on either U_{crit} or sprint velocity (both t<0.25, P>0.8), but exposure to 150 μmol l^{-1} dantrolene significantly reduced U_{crit} compared with controls (t=2.68, P<0.02), although it did not affect sprint velocity (t=–0.18, P=0.86; Fig. 4B).

Seven days after electroporation, the fish with elevated parv4 protein concentrations (GFP+parv4 vector) had significantly greater U_{crit} (t=–4.35, P<0.0001) and sprint velocity (t=2.49, P<0.02) compared with the fish that received the GFP-only vector (Fig. 5).

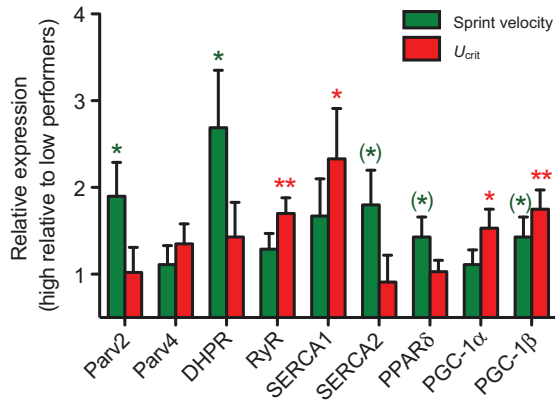

Fig. 2. Gene expression of high sprint velocity and U_{crit} performance fish relative to low performance fish. Significant differences are indicated by asterisks: $^{(*)}P$<0.10 (one-tailed P<0.05); *P<0.05; **P<0.01. Parv2, parvalbumin isoform 2; Parv4, parvalbumin isoform 4; DHPR, dihydropyridine receptor; RyR, ryanodine receptor; SERCA 1, sarcoplasmic reticulum Ca^{2+}-ATPase isoform 1; SERCA 2, sarcoplasmic reticulum Ca^{2+}-ATPase isoform 2; PPARδ, peroxisome proliferator activated receptor δ; PGC-1α, peroxisome proliferator activated receptor γ co-activator 1α; PGC-1β, peroxisome proliferator activated receptor γ co-activator 1β.

DISCUSSION

We provide the first experimental demonstration that parvalbumin has a direct effect on whole-animal locomotor performance. At the whole-animal level, sprint and sustained locomotion varied independently from each other, but some of the enabling mechanisms such as parvalbumin protein concentration had a positive effect on both. In contrast, several other traits including Ca^{2+} handling mechanisms (DHPR, RyR, SERCA) and metabolic regulators and enzymes had different effects on sprint and sustained locomotion. Differential expression of these enabling mechanisms can explain differences in locomotor performance between individuals.

Energy metabolism is often invoked to explain differences in locomotor performance (Gibb and Dickson, 2002). For example, aerobic scope – that is, the difference between resting and maximal rates of oxygen consumption – is a good predictor for sustained swimming in salmon (Eliason et al., 2011). However, metabolism does not always predict locomotor performance well (Gibb and Dickson, 2002). This may be partly due to the different components of metabolism, for example resting oxygen consumption and maximal activity of enzymes, that are used to predict sprint or sustained locomotion, and partly to conflicting demands for ATP that may or may not occur at the same time as locomotion (Clobert et al., 2000). Additionally, oxidative metabolism may be constrained by cardiovascular capacities that limit oxygen delivery to tissues at times of high demand (Steinhausen et al., 2008; Eliason et al., 2011).

In the case of our zebrafish, creatine kinase was a good predictor for sprint velocity. The phosphocreatine–creatine kinase system is responsible for relocating cellular ATP to sites of demand, and it is fast acting (Saks, 2008). This last characteristic may make it particularly important for sprint velocity, which requires a fast but not sustained ATP supply. Creatine kinase activity is heritable in stickleback (Garenc et al., 1998), and may thereby provide an evolutionary mechanism for selection of sprint performance. In contrast, and contrary to our predictions, citrate synthase and lactate dehydrogenase activities were not associated with increased locomotor performance. Citrate synthase activity is an indicator of mitochondrial capacity and abundance, and lactate dehydrogenase catalyses anaerobic ATP production. Our data indicate that neither constrains locomotor performance. However, ecological situations

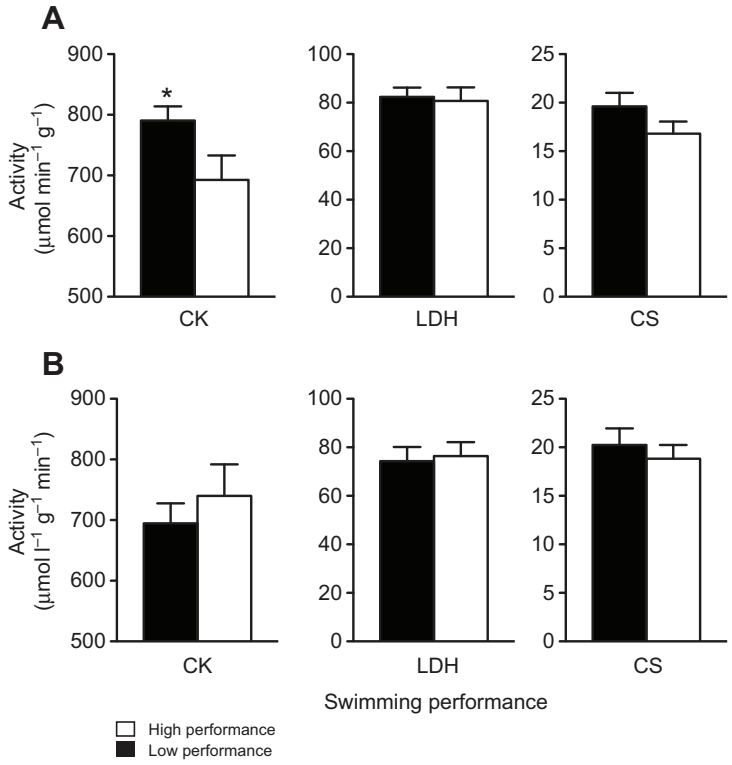

Fig. 3. Activities of metabolic enzymes (CK, creatine kinase; LDH, lactate dehydrogenase; CS, citrate synthase) of fish with high and low performance sprint (A) and U_{crit} (B). Significant differences are indicated by an asterisk (*P<0.05).

may arise where locomotion 'competes' with other ATP consuming functions such as growth, when maximal catalytical capacity may become limiting. Similarly, enzyme activity may become limiting in particular environmental conditions, such as low or high temperature environments (Guderley, 2004; Seebacher et al., 2010).

The differential expression of several transcriptional regulators in low and high performance groups indicates that metabolism does play a role in locomotion. PGC-1α is a well known regulator of metabolism (Scarpulla, 2008; Walter and Seebacher, 2009) that is induced by exercise to regulate several exercise-promoting functions ranging from angiogenesis to mitochondrial metabolism and gluconeogenesis (Rodgers et al., 2008; Chinsomboon et al., 2009; Seebacher and Glanville, 2010). PGC-1α also mediates slow-twitch muscle fibre expression (Lin et al., 2002a), and maintains muscle integrity (Sandri et al., 2006). Hence, the increased expression of PGC-1α in high sustained-exercise performance zebrafish is not surprising, and the expression level of this coactivator is likely to be an important mechanism in the evolution of endurance performance. It remains to be shown, however, whether expression levels are heritable (but see Le Moine et al., 2010) and, thus, confirm that its expression is a genetic mechanism underlying selection.

The nuclear receptor PPARδ is a target of PGC-1α that shifts mitochondrial substrate utilisation in skeletal muscle from carbohydrate to fatty acid oxidation (Constantin et al., 2007). In rats, this shift in substrate use is attended by a decrease in mitochondrial ATP production and an increase in phosphocreatine hydrolysis and anaerobic ATP production. At the same time, muscle fatigue resistance decreases (Constantin-Teodosiu et al., 2009). These characteristics can promote sprint performance and may explain the increased expression of PPARδ in the muscle of high sprint performance zebrafish. The role of PGC-1β is less defined than that of PGC-1α. In PGC-1β knock-out mice, the function of the mitochondrial electron transport chain is impaired, which is however partly compensated for by PGC-1α up-regulation (Lelliott

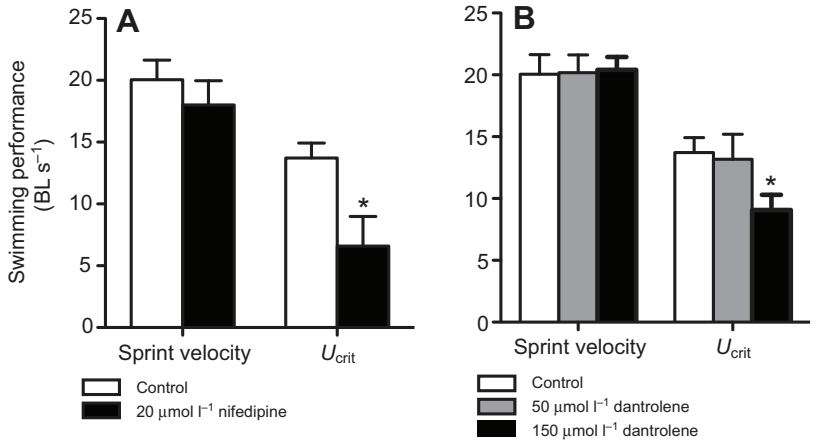

Fig. 4. The sensitivity of sprint velocity and U_{crit} to inhibition of dihydropyridine receptors (A, nifedipine treatment) and ryanodine receptors (B, dantrolene treatment). Significant differences are indicated by an asterisk (*P<0.05).

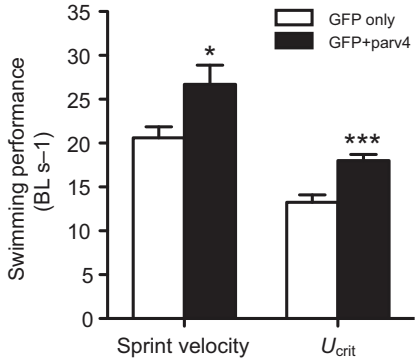

Fig. 5. Overexpression of parvalbumin 4 by *in vivo* electroporation (GFP+parv4) caused a significant increase in sprint velocity and in U_{crit} compared with a control in which only GFP was inserted into the fish genome (GFP only). Significant differences are indicated by asterisks (*P<0.05; ***P<0.0001).

et al., 2006). Hence, the action of PGC-1β appears to be broadly similar to that of PGC-1α in regulating a suite of processes in energy metabolism that may also include hepatic gluconeogenesis (Lin et al., 2002b). The increased expression of PGC-1α and PGC-1β indicates an overall enhancement of the metabolic machinery in high performance animals, where sustained locomotor performance is more sensitive than sprint performance to the action of PGC-1α.

Excitation–contraction coupling by DHPR and release of Ca^{2+} from stores in the sarcoplasmic reticulum *via* RyR have different effects on locomotor performance. We have shown here that sustained locomotor performance is more sensitive to pharmacological inhibition of either receptor than sprint performance. The reason may be that alternative Ca^{2+} release mechanisms from the sarcoplasmic reticulum (Dirkson, 2009; Rosenberg, 2009) are sufficient to provide the signal for brief sprint performance, but that these mechanisms cannot maintain the muscle function required for prolonged locomotion. In skeletal muscle, stimulation of RyR by DHPR occurs by direct interaction between these molecules. In contrast, in cardiac muscle, Ca^{2+} released by DHPR stimulates RyR (Dirkson, 2009). Nifedipine inhibits the Ca^{2+} release by DHPR but not the molecular interaction between DHPR and RyR (Fleckenstein, 1983). Hence, nifedipine treatment is more likely to affect U_{crit} *via* a depressing effect on cardiovascular performance than sprint performance.

The increased expression of RyR mRNA in high sustained performance fish points towards a direct relationship between mRNA concentration and RyR function. We found similar results in isolated rat muscle (James et al., 2011). Interestingly, however, in rat muscle RyR were associated with sprint performance (rates of muscle contraction and force production) rather than fatigue resistance (James et al., 2011). In contrast, DHPR mRNA concentrations in zebrafish were increased in high sprint performance animals, which seems contrary to our pharmacological results showing that sprint performance is relatively less sensitive to inhibition compared with sustained performance. The explanation may be that increased DHPR concentrations are important for sprint performance by improving excitation–contraction coupling, but that blocking DHPR mediated Ca^{2+} release affects U_{crit} by its depressing effect on cardiovascular performance as outlined above. That both DHPR and RyR are important for sprint performance is supported by the fact that zebrafish *relatively relaxed* mutants, in which densities of both receptors are severely reduced, have significantly slower sprint velocities (Hirata et al., 2007).

Endurance training caused an increase in DHPR and RyR protein concentrations in Atlantic salmon (*Salmon salar*) (Antilla et al., 2006), brown trout (*Salmon trutta*) (Antilla et al., 2008) and whitefish (*Coregonus lavaretus*) (Antilla et al., 2008). Exercise training has a pronounced effect on muscle physiology (Burgomaster et al., 2008) and may override phenotypic differences existing between untrained individuals. Additionally, DHPR and RyR expression may be associated with particular muscle fibre types, and DHPR and RyR concentrations are correlated with myosin heavy chain expression in rat muscle (Antilla et al., 2007). However, the response to exercise training was similar in red and white muscle of fish (Antilla et al., 2006; Antilla et al., 2008). Together, our findings and published data indicate that the function of both DHPR and RyR is important in determining locomotor performance. However, their relative roles in determining sprint and sustained locomotor performance in untrained animals differ, and these Ca^{2+} release mechanism are therefore a mechanism that can at least partly explain interindividual and interspecific differences in locomotor performance.

Muscle relaxation depends on the rate of Ca^{2+} re-sequestration into the sarcoplasmic reticulum, which is achieved by the activity of SERCA (Berchtold et al., 2000). Inhibition of SERCA activity in isolated rat muscle significantly decreased fatigue resistance (James et al., 2011), which indicates that replenishing Ca^{2+} stores is linked to sustained performance. Interestingly, our results here show that gene expression of the fast SERCA1 isoform is associated with high sustained performance, while expression of the slow SERCA2 isoform is increased in high sprint performance fish. We analysed whole-tail muscle, so we would expect both isoforms to be present, although we would also expect SERCA1 to be associated with increased sprint velocity. A possible explanation may be that more efficient (faster) slow twitch muscle may be advantageous for sprint performance, and *vice versa* for sustained performance (Lalli et al., 2001). SERCA activity is also strongly influenced by external regulators (East, 2000) such as phospholamban (Verboomen et al., 1992; Gustavssen et al., 2011), so the functional differences between the isoforms *per se* may be reduced.

Importantly, the efficacy of Ca^{2+} re-sequestration into the sarcoplasmic reticulum depends strongly on parvalbumin (Arif, 2008). Parvalbumin is an intracellular Ca^{2+} binding protein that is involved in numerous cellular processes by regulating Ca^{2+} concentration spatially and temporally (Arif, 2008). In muscle cells, parvalbumin binds Ca^{2+} with high affinity, thereby causing a rapid decrease in intracellular Ca^{2+} concentration that facilitates muscle relaxation in conjunction with SERCA, which subsequently pumps Ca^{2+} back into the sarcoplasmic reticulum (Berchtold et al., 2000). We provide here the first experimental evidence that locomotor performance is also influenced by parvalbumin concentration. At the level of isolated muscles, parvalbumin expression is positively associated with relaxation time (Münterer et al., 1995; Brownridge et al., 2009; Schoenman et al., 2010), and differences in relaxation time of different muscles within the same organism are correlated with differences in parvalbumin content (Wilwert et al., 2006; Brownridge et al., 2009). There may also be functional differences between different parvalbumin isoforms (Brownridge et al., 2009; Schoenman et al., 2010). In carp, there are eight parvalbumin isoforms that are differentially expressed in the anterior and posterior regions of the tail muscle, and which are associated with muscle relaxation rates (Brownridge et al., 2009). Zebrafish possess nine parvalbumin isoforms, but whether there are functional differences between these is not known (Friedberg, 2005). Our data show that overexpression of the parv4 isoform alone improved both sprint and

endurance performance. These data provide important insight into several questions regarding parvalbumin function (Berchtold et al., 2000). Firstly, that parvalbumin can be rate limiting for locomotor performance; secondly, that parvalbumin functions in both fast and slow twitch muscles; and thirdly, that a single isoform can be of wide-ranging functional significance. Our data are the first to bridge the gap between isolated muscle function and whole-animal locomotor performance. Hence, parvalbumin and the regulation of its expression are important mechanisms that may explain interindividual differences in locomotor performance as well as evolutionary patterns. The increased expression of parv2 in our zebrafish indicates that fast-twitch muscles and sprint performance are more sensitive to changes in parvalbumin concentration. This conclusion is supported by the preferential, albeit not exclusive, expression of parvalbumin in fast twitch, white muscle fibres (Hou et al., 1991; Schoenman et al., 2010).

We have identified a number of molecular traits that can explain differences in sprint and sustained locomotor performance between individuals. The importance of many of these mechanisms in influencing sprint or sustained locomotion is their relative expression or abundance rather than presence or absence. Understanding how these molecular traits function and what regulates their expression will therefore provide new insight into locomotion and its evolution, and into associated diseases (East, 2000; Berchtold et al., 2000). For example, the thermal sensitivity of parvalbumin Ca^{2+} binding affinity differs between species (Erickson et al., 2005). Our finding that parvalbumin has a direct influence on locomotor performance means that the chemical characteristics that modify the thermal sensitivity of parvalbumin binding affinity will also influence ecological performance. Evolutionary or reversible changes in the parvalbumin molecule (Kretsinger, 1980) can therefore be a mechanism underlying adaptation and maybe also acclimation of locomotor performance to different environmental conditions. Similar arguments can be made concerning the other traits we identify, such as RyR (Bellinger et al., 2008). The advantage of knowing specific molecular mechanisms underlying complex traits such as locomotion is that it shifts the evolutionary analysis of the complex trait into the sphere of bioinformatics with its increased power of analysis. Ecological and evolutionary studies should now progress to analysing promoter sequences and expression patterns of the traits we identified, and doubtlessly others, to identify evolutionary patterns and constraints.

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