The Journal of Experimental Biology 215, 746-759

doi:10.1242/jeb.065425

RESEARCH ARTICLE

Mechanisms underlying parallel reductions in aerobic capacity in non-migratory threespine stickleback (*Gasterosteus aculeatus*) populations

Anne C. Dalziel*, Michelle Ou and Patricia M. Schulte

Biodiversity Research Center and Department of Zoology, 6270 University Blvd, University of British Columbia, Vancouver, BC, Canada V6T 1Z4

*Author for correspondence (dalziel@zoology.ubc.ca)

Accepted 10 November 2011

SUMMARY

Non-migratory, stream-resident populations of threespine stickleback, *Gasterosteus aculeatus*, have a lower maximum oxygen consumption ($\dot{M}_{O2,max}$) than ancestral migratory marine populations. Here, we examined laboratory-bred stream-resident and marine crosses from two locations (West and Bonsall Creeks) to determine which steps in the oxygen transport and utilization cascade evolved in conjunction with, and thus have the potential to contribute to, these differences in $\dot{M}_{O2,max}$. We found that West Creek stream-resident fish have larger muscle fibres (not measured in Bonsall fish), Bonsall Creek stream-resident fish have smaller ventricles, and both stream-resident populations have evolved smaller pectoral adductor and abductor muscles. However, many steps of the oxygen cascade did not evolve in stream-resident populations (gill surface area, hematocrit, mean cellular hemoglobin content and the activities of mitochondrial enzymes per gram ventricle and pectoral muscle), arguing against symmorphosis. We also studied F1 hybrids to determine which traits in the oxygen cascade have a genetic architecture similar to that of $\dot{M}_{O2,max}$. In West Creek, $\dot{M}_{O2,max}$, abductor and adductor size all showed dominance of marine alleles, whereas in Bonsall Creek, $\dot{M}_{O2,max}$ and ventricle mass showed dominance of stream-resident alleles. We also found genetically based differences among marine populations in hematocrit, ventricle mass, pectoral muscle mass and pectoral muscle pyruvate kinase activity. Overall, reductions in pectoral muscle mass evolved in conjunction with reductions in $\dot{M}_{O2,max}$ in both stream-resident populations, but the specific steps in the oxygen cascade that have a genetic basis similar to that of $\dot{M}_{O2,max}$, and are thus predicted to have the largest impact on $\dot{M}_{O2,max}$, differ among populations.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/5/746/DC1

Key words: maximum oxygen consumption, common garden experiment, F1 hybrids, symmorphosis, pectoral muscle.

INTRODUCTION

Maintaining a high capacity for aerobic exercise can be an important contributor to Darwinian fitness (reviewed by Husak and Fox, 2008; Irschick et al., 2008). One trait that can limit the capacity for aerobic exercise is an animal's maximum capacity for aerobic energy metabolism (Bennett, 1989; Bennett, 1991), typically measured as maximum oxygen consumption ($\dot{M}_{O2,max}$). In spite of high phenotypic plasticity, $\dot{M}_{O2,max}$ can be heritable (e.g. Garland and Bennett, 1990; Nespolo et al., 2005) and experience selection in wild populations (e.g. Hayes and O'Connor, 1999; Jackson et al., 2001). In addition, selection studies in rodents have shown that aerobic capacity can evolve very rapidly (Henderson et al., 2002; Rezende et al., 2006; Sadowska et al., 2008; Gębczyński and Konarzewski, 2011). Mechanistically, $\dot{M}_{O2,max}$ is determined by a variety of structures and processes related to oxygen uptake from the environment, transport to the working muscles and utilization by the mitochondrial electron transport chain (reviewed by Taylor and Weibel, 1981; Wagner, 1996). However, the relative importance of each step in this oxygen cascade in determining $\dot{M}_{O2,max}$ may vary with an individual's physiological status (reviewed by Wagner, 1996; Wagner, 2010), and among individuals, populations and species (e.g. Frappell et al., 2002).

Interspecific comparisons of metabolically active species with less athletic species have shown that differences in $\dot{M}_{O2,max}$ are often associated with multiple changes in the oxygen transport and utilization cascade (reviewed by Suarez, 1996; Hoppeler and Weibel, 1998; Bernal et al., 2001; Turner et al., 2006). These interspecific studies suggest that the oxygen transport and utilization cascade evolves in a 'symmorphotic' fashion, such that all steps in the oxygen cascade are well matched in capacity, and no single trait sets the upper limit for $\dot{M}_{O2,max}$ (sensu Taylor and Weibel, 1981). However, evolutionary theory (e.g. Garland, 1998; Hansen et al., 2006) and recent intraspecific empirical studies (e.g. Gonzalez et al., 2006; Chappell et al., 2007; Gębczyński and Konarzewski, 2011) argue that this is not the case. In particular, experimental evolution studies in rodents suggest that interspecific comparisons simply miss the step-wise evolution of aerobic capacity that can be observed at shorter time scales (e.g. Henderson et al., 2002; Howlett et al., 2009; Kirkton et al., 2009; Gębczyński and Konarzewski, 2011). However, little is known about how maximum aerobic performance and underlying traits have evolved among natural populations that experience more complex selective environments, relative to experimental evolution studies, with multiple divergent and concurrently acting sources of selection (but see Odell et al., 2003). Determining which step(s) underlie differences in $\dot{M}_{O2,max}$ among natural populations can provide mechanistic information about the traits limiting $\dot{M}_{O2,max}$ in a particular species, provide insight into the costs of having a high $\dot{M}_{O2,max}$, and establish the pace at which

this ecologically relevant trait evolves in the wild. When studying these questions, intraspecific comparisons among closely related populations may be preferable to interspecific studies, because there is a reduced likelihood of divergence in traits not mechanistically related to $\dot{M}_{O2,max}$ (reviewed by Langerhans and Reznick, 2009). In addition, individuals with genetically based differences in performance capacity can be inter-bred (e.g. Seiler and Keeley, 2007; Rouleau et al., 2010), which allows the use of forward genetic approaches to identify and test the impacts of 'candidate' traits predicted to contribute to differences in performance (Feder et al., 2000; Dalziel et al., 2009).

Threespine stickleback (*Gasterosteus aculeatus* Linnaeus 1758) are small teleost fish found across the Northern Hemisphere (reviewed by Bell and Foster, 1994; Ostlund-Nilsson et al., 2007). In British Columbia, Canada, multiple freshwater populations of threespine stickleback evolved from marine ancestors after the Cordilleran ice sheet receded, approximately 10,000–12,000 years ago (reviewed by McPhail, 1994). Since this time, freshwater stickleback populations have diverged from each other, and from their marine ancestors, in a number of morphological, behavioural and physiological traits (reviewed by McKinnon and Rundle, 2002; Ostlund-Nilsson et al., 2007), but crosses between divergent populations are still possible. The multiple independent colonizations of freshwater streams also allows for the study of replicate evolutionary events, which can provide insight into the influence of selection on trait evolution (reviewed by Schluter et al., 2010; Losos, 2011). We have previously shown that two populations of stream-resident stickleback in British Columbia have evolved reductions in maximum oxygen consumption ($\dot{M}_{O2,max}$) compared with sympatric anadromous-marine populations (hereafter referred to as 'marine') (Dalziel et al., 2012). Wild stream-resident stickleback from Belgium also show reductions in $\dot{M}_{O2,max}$ (Tudorache et al., 2007), which indicates that reductions in maximum oxygen consumption have evolved rapidly (<12,000 years) and in parallel after freshwater colonization, and is suggestive of a role for selection in trait loss (Lahti et al., 2009).

Here, we examine a number of steps in the oxygen transport and utilization cascade to determine the mechanistic basis for the reductions in $\dot{M}_{O2,max}$ in stream-resident stickleback. Because many traits in the oxygen cascade are phenotypically plastic in fish (e.g. Hoffmann and Borg, 2006; Farrell et al., 1991; Henriksson et al., 2008), we have used fish raised in a common laboratory environment to minimize the effects of phenotypic plasticity (Dalziel et al., 2012). Specifically, we measured traits related to the capacity for oxygen uptake from the water (gill surface area), oxygen transport to the working muscle (blood haemoglobin concentration, hematocrit and ventricle size), oxygen diffusion from the blood to the mitochondria (muscle fibre size) and oxygen use at the mitochondria within the swimming muscles (pectoral muscle size and muscle aerobic capacity, which we assessed by measuring fibre-type composition and the activities of mitochondrial enzymes as proxies for mitochondrial content). We predicted that the traits that are the most costly to maintain, either directly or because of trade-offs with other functions, such as maintaining ion gradients (e.g. a high gill surface and small diameter muscle fibres), would evolve most rapidly in stream-resident fish.

MATERIALS AND METHODS

Experimental animals

The threespine stickleback used in this study were collected, bred and raised by Dalziel et al. (Dalziel et al., 2012). Briefly, our crosses were made from fish collected at Bonsall Creek on Vancouver Island and West Creek on mainland British Columbia (Fig. 1) (BC Ministry of Environment Fish Collection Permits NA/SU06-26169 and NA/SU07-38414). In this experiment, we studied 22 crosses made from Bonsall Creek parents [five pure stream (SS), six pure marine (MM), six stream mother × marine father (SM) and five marine mother × stream father (MS)] and 17 crosses from West Creek parents (five SS, six MM, three SM and three MS). Fish were raised in de-chlorinated Vancouver tap water brought to 2±0.5 p.p.t. with Instant Ocean® sea salt (Aquarium Systems Inc., Mentor, OH, USA), and were fed live brine shrimp twice per day for their first month, *Daphnia* and bloodworms daily for the next 3 months, and *Mysis* shrimp and bloodworms (chironomid larvae) from 4 months on. Each cross was raised in a separate 30 gallon tank and families were reduced to 20 fish per tank at the age of 2 months to keep all crosses at similar densities. Fish were reared at a natural photoperiod and at laboratory temperatures (~11–17°C) until March (~9–11 months of age). At this point they were transferred to a 15°C environmental chamber with a controlled 12 h:12 h light:dark photoperiod (the natural photoperiod for our collection sites in March) to prevent fish from entering the reproductive state. The maximum aerobic metabolic rates (MMR or $\dot{M}_{O2,max}$; measured as $\mu mol\, O_2\, g^{-1}\, h^{-1}$) of these fish were originally presented in Dalziel et al. (Dalziel et al., 2012). We used exercise to induce $\dot{M}_{O2,max}$ because stickleback are highly motivated by the presence of the experimenter and swim well in experimental flumes (Taylor and McPhail, 1986; Dalziel et al., 2012). Although $\dot{M}_{O2,max}$ can be reached post-prandially in some sluggish species of fish, such as cod, swimming results in higher estimates of $\dot{M}_{O2,max}$ in more active fishes, such as salmonids (reviewed in Claireaux et al., 2005). After collecting data for the measures described in Dalziel et al. (Dalziel et al., 2012), fish were left to acclimate at 15°C and 12 h:12 h light:dark for at least another month before they were terminally sampled for the biochemical measures presented in this paper. We found that a related physiological trait, prolonged swimming performance (U_{crit}), is highly repeatable after 1 month (r^2=0.901, P<0.001) (Dalziel et al., 2012), indicating that the 1 month interval between assessment of $\dot{M}_{O2,max}$ and the underlying traits should not significantly affect our interpretations. At the time of sampling, the majority of crosses (31 of 39) were approximately 1 year and 5–9 months of age, but eight West Creek crosses (three SS, three MM, one SM and one MS) were approximately 2.5 years of age. No fish displayed evidence of senescence at the time of sampling (A.C.D., personal observations).

Collection of blood and tissue samples

Stickleback were killed by placing each fish in a container of tank water with an overdose of anaesthetic ($1\, g\, l^{-1}$ tricaine methanesulfonate buffered with $2\, g\, l^{-1}$ sodium bicarbonate). As soon as a fish lost equilibrium (<30 s), it was removed from the anaesthetic, blotted dry and weighed. To collect blood, we severed the caudal peduncle with a razor blade just posterior to the cloaca and collected blood in two capillary tubes for the determination of hemoglobin concentration [Hb] and hematocrit (Hct) (see 'Analysis of blood and tissue samples' for further information). We then dissected out the heart, pectoral adductor and pectoral abductor muscles with the aid of a dissecting microscope. All tissues were snap-frozen in liquid N_2 and stored at –80°C. We next removed the left gill basket, rinsed the gills with distilled water, added the gills to Karnovsky's fixative (2.5% glutaraldehyde and 2% formaldehyde in $0.1\, mol\, l^{-1}$ sodium phosphate buffer, pH 7.2) and stored samples at 4°C.

We used a second set of fish from the same crosses to collect pectoral muscle samples for histological measurements. These fish

were also terminally anaesthetized with an overdose of anaesthetic (as before), and after they lost equilibrium (<30 s), the fish were removed from the anaesthetic, blotted dry and weighed. The full left pectoral girdle was removed from the fish, coated in mounting medium (Fisher Histoprep; Fisher Scientific, Nepean, ON, Canada) and placed on a cork disk at the pectoral fin insertion point with the pectoral muscle perpendicular to the disk plane. This muscle preparation was rapidly frozen in 2-methylbutane (cooled in liquid N_2), wrapped in foil and stored at –80°C.

We were unable to collect data from all of our crosses for every measurement. In particular, we do not have gill morphology data for any West Creek crosses, or histological data for any Bonsall Creek crosses. The University of British Columbia Animal Care Committee approved all breeding and experimental procedures (A07-0288).

Analysis of blood and tissue samples

Blood [Hb], Hct and mean cellular hemoglobin content

We collected Hct samples in heparanized micro-hematocrit capillary tubes (Fisher Scientific), sealed the tubes, stored them on ice until a dissection was complete, and then centrifuged the capillary tubes at 5000 ***g*** for 3 min and recorded Hct. To measure blood [Hb], we collected blood in 5 µl micro blood collecting tubes (Fisher Scientific), immediately added this blood sample to 1 ml of Drabkins' reagent (Sigma-Aldrich, Oakville, ON, Canada), and stored samples at 4°C. We measured [Hb] spectrophotometrically at A_{540} (Blaxhall and Daisley, 1973), and calculated mean cellular hemoglobin content (MCHC) as [Hb]/Hct.

Gill morphometrics

Our measurements of gill morphology followed Hughes (Hughes, 1984), and were conducted using a Leica MZ16A stereo-microscope (Leica Microsystems GmbH, Wetzlar, Germany). Measurements were made in ImageJ (Rasband, 2011). We used the second gill arch to conduct all measurements. There was variation in filament length along a given gill arch, so each arch was divided into three equal sections (anterior, middle and posterior). We counted the total number of filaments in each section and measured the length of three randomly chosen filaments in each of the three sections (11× magnification). The linear spacing between lamellae along the filaments was determined by measuring the distance covered by 10 lamellae on six randomly chosen filaments (50× magnification). Lamellar area was calculated by breaking lamellae off the filaments and measuring the area of five randomly chosen lamellae per sample (115× magnification). Total gill surface area (A) was calculated as $A=LnB$, where L is the total filament length (mm) on all four gill arches (each with two rows of filaments), n is the number of lamellae per millimeter on both sides of the filament, and B is the average bilateral surface area of the lamellae (mm^2).

Ventricle and pectoral muscle masses and enzyme activities

Frozen ventricles and pectoral adductor and pectoral abductor muscles were weighed and immediately added to 20 volumes (pectoral muscles) or 79 volumes (ventricles) of chilled homogenization buffer (50 mmol l^{-1} Hepes, 1 mmol l^{-1} EDTA and 0.1% Triton X-100, pH 7.4), and homogenized in 4 ml Wheaton glass homogenizers kept on ice. We measured enzyme activities for cytochrome *c* oxidase (COX; EC 1.9.3.1, complex IV in the electron transport chain), citrate synthase (CS; EC 2.3.3.1, a citric acid cycle enzyme), creatine phosphokinase (CPK; EC 2.7.3.2, an enzyme that reversibly catalyzes the transfer of phosphate between ATP and creatine phosphate, a compound that stores energy and facilitates intracellular ATP transfer), pyruvate kinase (PK; EC 2.7.1.40, an enzyme in the glycolytic pathway) and lactate dehydrogenase (LDH; EC 1.1.1.27, an enzyme that catalyzes the inter-conversion of pyruvate and NADH to lactate and NAD+, which is crucial for replenishing NAD+, and maintaining high glycolytic flux during cellular hypoxia) on whole-cell extracts from the ventricle, pectoral adductor and pectoral abductor at 25°C (e.g. Moyes et al., 1997; McClelland et al., 2005). We optimized all assays to ensure that substrates were not limiting and modified protocols for measurement using a plate spectrophotometer (Spectramax 190, Molecular Devices, Sunnyvale, CA, USA). Final concentrations for each assay were as follows: COX (50 µmol l^{-1} reduced cytochrome *c*, 0.5% Tween 20, in 50 mmol l^{-1} Tris, pH 8), CS (0.15 mmol l^{-1} DTNB, 0.15 mmol l^{-1} Acetyl CoA and 0.5 mmol l^{-1} oxalacetic acid in 50 mmol l^{-1} Tris, pH 8), PK (0.15 mmol l^{-1} NADH, 5 mmol l^{-1} ADP, 100 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 10 µmol l^{-1} fructose 1,6-bisphosphate, 50 U ml^{-1} LDH, 5 mmol l^{-1} phosphoenol pyruvate in 50 mmol l^{-1} Tris, pH 7.4), LDH (5 mmol l^{-1} NADH, 25 mmol l^{-1} pyruvate in 50 mmol l^{-1} Tris, pH 7.4) and CPK (50 mmol l^{-1} creatine phosphate, 3 mmol l^{-1} ADP, 1.5 mmol l^{-1} NADP+, 20 mmol l^{-1} glucose, 12 mmol l^{-1} AMP, 25 mmol l^{-1} $MgCl_2$, 2 U $µl^{-1}$ Hexokinase, 1.5 U ml^{-1} GPDH in 50 mmol l^{-1} Tris, pH 7.4). We assayed COX on fresh homogenates that had been kept on ice for less than 1 h, and measured all other enzymes and total protein on tissue homogenates that were frozen once at –80°C, thawed and kept on ice for less than 2 h, because preliminary experiments indicated that this approach maximized enzyme activities. All samples were assayed in triplicate, and background reaction rates (no substrate present) were subtracted for CS, PK, LDH and CPK. We measured the protein content in all tissue homogenates with Bradford Reagent (Sigma-Aldrich), and excluded samples with protein concentrations that were greater than one standard deviation from the tissue average protein content. Protein concentrations in homologous tissues were similar in all cross-types (data not shown).

Muscle histology

In addition to studying muscle metabolic characteristics by measuring the activities of metabolic enzymes predicted to reflect muscle fibre-type composition, we also directly examined fibre-type composition of pectoral adductor and abductor muscles in our West Creek crosses. Generally, fish have three types of muscle fibres: red or slow-twitch fibres (small fibres with high oxidative and low glycolytic capacities, similar to mammalian type I fibres), pink or intermediate fibres (intermediate-sized fibres with high oxidative and high glycolytic capacities, similar to mammalian type IIA/IIX fibres), and white or fast-twitch fibres (large fibres with low oxidative and high glycolytic capacities, similar to mammalian type IIB fibres) (reviewed by Johnston et al., 2011). Much more is known about the metabolic differences found among mammalian fibre types, so we also incorporated knowledge about homologous mammalian fibres when making our predictions (reviewed by Zierath and Hawley, 2004). We predicted that stickleback red fibres would have high CS and COX activities (which we use as proxies for aerobic capacity), pink fibres would have similar or slightly lower CS and COX activities, and white fibres would have the lowest activities of these mitochondrial enzymes. Red fibres were also predicted to have the lowest activities of LDH (which we use as a proxy for glycolytic potential in oxygen-limited conditions), PK (a proxy for glycolytic potential) and CPK (a proxy for phosphocreatine levels). White fibres are predicted to have the highest activities of CPK of all three fibre types. Our predictions for PK and LDH activities in white and pink fibres were less clear. The mammalian

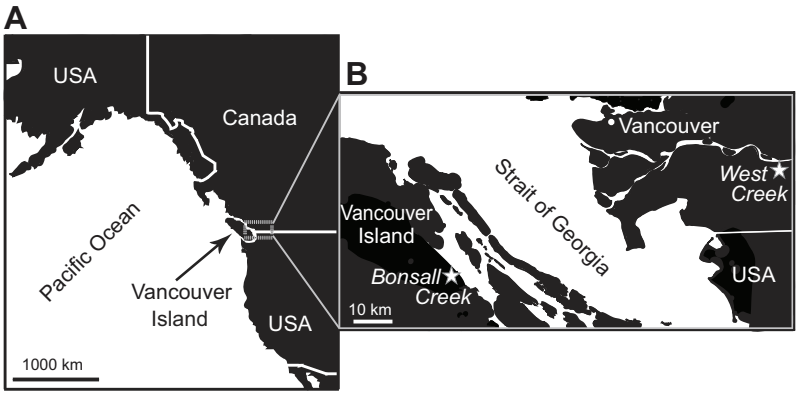

Fig. 1. Locations of the threespine stickleback populations used in this study. (A) Western North America, with the sampling area outlined with a grey hatched square; (B) region within the grey hatched square, with stickleback collection sites marked with white stars and labeled in italics.

literature suggests that white fibres should have the highest activities of PK and LDH, but in carp (*Cyprinus carpio*), pink fibres have LDH activities that are higher (Johnston et al., 1977) or similar to those of white fibres (Jabarsyah et al., 2000). Te Kronnie et al. (Te Kronnie et al., 1983) found that the axial musculature of threespine stickleback is primarily composed of white fibres, with some pink fibres and no red fibres. To date, the fibre-type composition of pectoral abductors and adductors had not been separately studied due to their small size and similar 'red' phenotype (e.g. Guderley and Couture, 2005; Orczewska et al., 2010). We examined the fibre-type composition of the pectoral muscles used for prolonged swimming (Taylor and McPhail, 1986; Walker, 2004) as a proxy for muscle mitochondrial content, which can influence maximum oxygen utilization potential.

Frozen pectoral muscle samples were sectioned (10 μm) transverse to fibre length in a –20°C cryostat (Leica CM3050 S, Leica Microsystems, Nussloch, Germany), and sections for histological analysis were taken at the midpoint of the pectoral muscle. These sections were transferred to glass slides and stored at –80°C. Sections were stained for succinate dehydrogenase (SDH) activity following the protocols of Scott et al. (Scott et al., 2009), and then photographed using light microscopy (Olympus FSX100, Tokyo, Japan). We measured the cross-sectional area of at least 20 white, pink and red muscle fibres in the pectoral adductor muscle and 20 of the largest and 20 of the smallest red fibres in the abductor muscles to quantify muscle size heterogeneity in the adductor, with ImageJ. We also measured the relative area of pink, white and red fibres in the abductor muscle (there are no white fibres in the adductor muscle) using ImageJ. We attempted to calculate the capillary density in pectoral muscles with an alkaline phosphatase stain. We verified this method for stickleback (A.C.D. and R. H. Dhillon, unpublished) and *Fundulus heteroclitus* caudal steaks (Dhillon and Schulte, 2011), but were unable to reliably quantify capillary density in pectoral muscle samples, possibly because capillaries in stickleback pectoral muscles are very tortuous (A.C.D., unpublished).

Statistical analysis

All statistical analyses were conducted using R v2.11.1 (R Development Core Team, 2010). We first tested for effects of fish size on all of our measurements. If measurements were significantly correlated with mass, we corrected for size by calculating the residuals from a least-squared regression against mass. In the case of $\dot{M}_{O_2,max}$, we calculated residuals from a least-squared regression of $\log_{10}\dot{M}_{O_2,max}$ against $\log_{10}$mass because $\dot{M}_{O_2,max}$ is normally found to scale exponentially with size (reviewed by Weibel and Hoppeler, 2005), and this transformation linearizes the data. We used untransformed data for all other measurements because we had no *a priori* hypothesis for these relationships, and linear regressions provided good fits to the data. To test the influence of cross-type on our variables, we used a mixed-effects model with individual nested within family (random effects) and family nested within cross-type (fixed effect) with the nlme package in R (Pinheiro et al., 2009). All data met the assumptions of homogeneity of variance and normality. Tukey's honestly significant difference *post hoc* tests were used to detect pairwise differences using the multcomp package in R (Hothorn et al., 2008). The number of families measured for each variable is listed in the figure or table legends, and the number of individuals per family sampled is as follows: for blood variables (Hct, [Hb] and MCHC), we measured at least three males and three females from each family; for tissue masses, we sampled at least three females per family; for histological measures, we sampled at least two females per family; and for measurements of enzyme activity, we sampled at least two females per family. Our data are not paired at the level of the individual, so comparisons among traits can only be made at the levels of family and cross-type. However, because individual data are nested within family, individual variability is incorporated into our mixed-effects models.

The focus of this paper is on the effect of ecotype (i.e. stream resident *vs* marine) and not sex, so we focused on female fish for the majority of our measures (histology, enzymes and tissue masses). However, we did collect sufficient data from both sexes for gill morphology and blood variables and for these measures we present data for both sexes and divide cross-types into male and female groups in statistical analyses. For the remainder of our measured variables (histological data, tissue masses and enzyme activities), we did not collect a sufficient number of males from all cross-types so we present data for, and performed statistical tests on, females only. In the cases where we were able to measure enough males to also investigate the effect of sex in a subset of cross-types (ventricle and pectoral muscle mass, and pectoral enzyme activities), we present male and female data in supplementary material Figs S1–3 and Table S1, and have highlighted any significant effects of sex in the main text.

We also explicitly tested for an effect of location (i.e. Bonsall *vs* West Creeks) by using a mixed-effects model with location as a fixed effect and cross-type, family and individual as nested random effects. Differences in dominance between West and Bonsall Creeks crosses were detected by modifying our original statistical model into a genetic model with terms for additivity, dominance, location and interactions between degree of dominance and location (y=location+additive+dominance+location×dominance) with the nlme package in R (Pinheiro et al., 2009). Differences in dominance between Bonsall and West Creek were tested by examining the interaction between location and dominance. We did not use

Table 1. Gill morphology of laboratory-bred F1 threespine stickleback families from Bonsall Creek parents

	Mass	Total number of filaments	Mean filament length (mm) for each gill section	Total filament length (mm)	Residual total filament length	Lamellar spacing (lamellae mm^{-1} filament)	Lamellar area (μm^2)	Raw gill surface area (cm^2)	Residual total gill surface area
Bonsall stream Female (*N*=3 families)	2.24±0.52	41.8±0.4	1.063±0.139 1.620±0.342 0.931±0.090	111.68±12.72	–15.75±0.17^{b}	55.42±0.52	15.53±0.89	38.73±4.92	–2.15±1.80
Bonsall stream Male (*N*=4 families)	1.99±0.26	43.2±0.9	1.071±0.022 1.848±0.071 0.967±0.076	120.40±7.02	–0.86±2.82^{a,b}	55.83±1.04	16.02±1.35	43.73±3.85	5.43±3.76
Bonsall hybrids Female (*N*=10 families)	2.09±0.19	43.4±0.8	1.159±0.076 1.917±0.093 0.962±0.031	123.55±7.14	–0.16±3.57^{a,b}	57.04±1.05	13.70±1.03	38.82±4.53	–0.50±3.33
Bonsall hybrids Male (*N*=10 families)	2.13±0.16	41.4 ±0.8	1.138±0.062 1.897±0.100 1.118±0.084	122.75±6.54	–1.85±4.90^{a,b}	54.68±0.75	12.05±0.68	32.48±2.65	–7.22±2.23
Bonsall marine Female (*N*=5 families)	1.69±0.10	44.6±0.8	1.069±0.060 1.841±0.068 1.057±0.041	124.42±7.13	10.28±6.90^{a}	56.80±1.11	14.21±0.33	40.23±2.79	4.88±2.96
Bonsall marine Male (*N*=6 families)	1.73±0.13	43.3±0.7	1.068±0.064 1.814±0.065 1.077±0.038	120.59±3.40	5.52±2.48^{a}	57.3±0.43	13.83±0.53	38.28±1.42	2.54±1.87

Data are presented as the grand means ± s.e.m. of all family means, but statistical tests included all individuals in a nested ANOVA model (see Results). There was a significant effect of mass on filament length, so residual filament lengths were used for statistical tests, but raw data are also presented here for clarity. Different superscripted letters indicate significant differences among groups for a given measure (P<0.05). If letters are not included, there were no significant differences among the six groups.

correlation analysis to detect associations between $\dot{M}_{O2,max}$ and candidate morphological and physiological traits because of the statistical problems that arise when using non-independent F1 hybrids. Although our data cannot provide causal evidence that a given trait, or set of traits, is responsible for decreases in $\dot{M}_{O2,max}$, our experimental design does allow us to reject the hypothesis of a strong functional linkage for candidate traits that did not evolve as predicted, or did not have a genetic architecture similar to that of prolonged swimming performance.

RESULTS

Gill morphology (both sexes; Bonsall Creek crosses only)

There were no significant differences among cross-types in filament number ($F_{5,30}$=2.215, P=0.0788), lamellar spacing ($F_{5,30}$=1.134, P=0.3639) or bilateral lamellar area ($F_{5,30}$=2.2816, P=0.0716). There were significant difference in total filament length among cross-types ($F_{5,30}$=2.737, P=0.0375), but this variation did not result in significant differences in overall gill surface area among Bonsall Creek laboratory-raised crosses ($F_{5,30}$=2.475, P=0.0543; Table 1).

Hct, [Hb] and MCHC (both sexes; Bonsall and West Creek crosses)

We found significant differences in Hct among cross-types ($F_{11,271}$=4.989, P<0.0001; Table 2). All differences detected by *post hoc* tests were between West and Bonsall Creek crosses, suggesting an effect of location and not ecotype. The effect of location was significant in our mixed-effects model explicitly testing this (t_{10}=5.595, P=0.0002). There were also significant differences in [Hb] among cross-types ($F_{11,280}$=4.5744, P=0.0001; Table 2), but differences in [Hb] are influenced by Hct, as, all else being equal, samples with a higher Hct will have a higher [Hb] for a given volume of blood. When corrected for Hct, we found no differences in [Hb] per red blood cell (MCHC) ($F_{11,255}$=2.092, P=0.0213; Table 2). We also conducted preliminary measurements of whole-blood-cell haemoglobin P_{50} (Hb P_{50}) from wild-caught Bonsall Creek marine and stream-resident fish, and did not find significant differences among ecotypes (see supplementary material Fig. S1).

Ventricle mass and enzyme activities (females only; Bonsall and West Creek crosses)

We found significant differences in residual ventricle mass among cross-types, with Bonsall Creek marine crosses having significantly larger hearts than those of all other crosses except West Creek marine crosses ($F_{5,30}$=3.460, P=0.0138; Fig. 2). Although West Creek marine fish had slightly larger ventricles than did stream-resident fish from West Creek, this difference was not significant. We did not find a significant effect of location (t_4=–0.887, P=0.4253; females only) or sex ($F_{1,6}$=0.4487, P=0.5278) on ventricle mass (see supplementary material Fig. S2).

We measured the activities of COX and CS as markers for mitochondrial content, PK and LDH as markers for glycolytic capacity, and CPK as a marker for ATP transfer potential in the ventricle. We found no significant differences among cross-types for CS ($F_{5,30}$=2.0557, P=0.0991), PK ($F_{5,30}$=0.8933, P=0.4981), LDH ($F_{5,30}$=1.9132, P=0.1216) or CPK ($F_{5,30}$=1.370, P=0.2634) activity per gram ventricle (Table 6). We did find significant differences among cross-types for COX activity per gram ventricle ($F_{5,30}$=8.1045, P<0.001), and *post hoc* tests indicated that five of the six significant differences were between West and Bonsall Creek crosses. The effect of location on ventricle COX activity was significant (t_4=2.8035, P=0.0486), with West Creek stream-resident and hybrid fish having a slightly higher activity of COX per gram ventricle than all Bonsall cross-types, and also West Creek marine crosses.

Table 2. Blood hematocrit (Hct), hemoglobin concentration ([Hb]) and mean cellular hemoglobin content (MCHC) of laboratory-bred F1 threespine stickleback families from Bonsall and West Creek parents

	[Hb] (mmol l^{-1})	Hct (% RBC)	MCHC ([Hb]/Hct)
Bonsall stream	(*N*=5)	(*N*=5)	(*N*=5)
Female	1.29±0.14^{a,d}	38±3^{c,d}	3.42±0.14
Male	1.48±0.12^{b,c}	41±3^{a,b,c,d}	3.69±0.17
West stream	(*N*=5)	(*N*=5)	(*N*=5)
Female	1.59±0.07^{a,b,c,d}	49±2^{a,b}	3.23±0.09
Male	1.56±0.09^{a,b,c,d}	49±3^{a,b}	3.23±0.05
Bonsall hybrid	(*N*=10)	(*N*=11)	(*N*=10)
Female	1.33±0.05^{a,b,c,d}	42±2^{b,c,d}	3.20±0.07
Male	1.44±0.04^{a,b,c,d}	45±1^{a,b,c}	3.28±0.09
West hybrid	(*N*=5)	(*N*=5)	(*N*=5)
Female	1.64±0.13^{a,b,c,d}	49±1^{a,b}	3.35±0.27
Male	1.57±0.09^{a,b,c,d}	46±2^{a,b,c}	3.44±0.31
Bonsall marine	(*N*=5)	(*N*=5)	(*N*=5)
Female	1.27±0.08^{a,c}	37±2^{d}	3.34±0.10
Male	1.44±0.06^{b,d}	43±2^{a,b,c}	3.36±0.09
West marine	(*N*=6)	(*N*=6)	(*N*=6)
Female	1.67±0.09^{a,b,c,d}	51±1^{a}	3.30±0.17
Male	1.64±0.19^{a,b,c,d}	47±3^{a,b}	3.53±0.20

Values are the grand means ± s.e.m. of all family means, but statistical tests included all individuals in a nested ANOVA model (see Results). *N* is the number of families sampled for each cross-type. Different superscripted letters indicate significant differences among groups for a given measure (P<0.05). If letters are not included, there were no significant differences among the 12 groups. RBC, red blood cell.

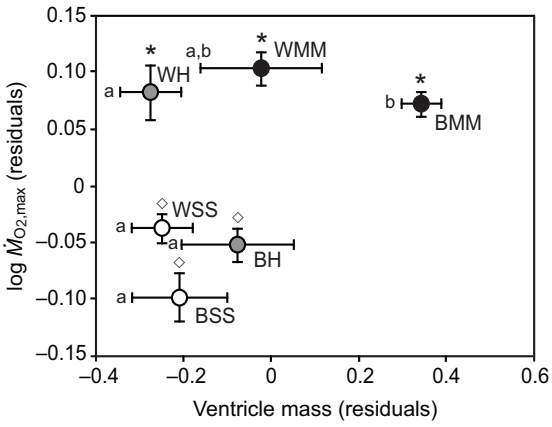

Fig. 2. Residual ventricle mass of laboratory-bred F1 threespine stickleback females from Bonsall and West Creek parents plotted against the residuals of log maximum oxygen consumption ($\dot{M}_{O2,max}$). Pure stream × stream crosses (SS; *N*=5 Bonsall families and 5 West Creek families) are indicated with white circles, pure marine × marine (MM; *N*=6 Bonsall families and 4 West Creek families) are indicated by solid black circles, and hybrid crosses (H; *N*=11 Bonsall families and 5 West Creek families) are indicated with grey circles. The collection location is also noted next to each data point: B, Bonsall Creek; W, West Creek. Data are presented as the grand means ± s.e.m. of all family means, but statistical tests included all individuals in a nested ANOVA model (see Results). Different letters indicate significant differences among the six cross-types for residual ventricle mass (P<0.05); different symbols indicate significant differences among cross-types for the residuals of log$\dot{M}_{O2,max}$ (P<0.05) (from Dalziel et al. 2012).

Pectoral muscle mass, fibre type (females only; West Creek crosses only) and enzyme activities (females only; Bonsall and West Creek crosses)

We found significant differences in residual adductor ($F_{5,32}$=14.499, P<0.0001) and abductor mass ($F_{5,32}$=11.251, P<0.0001) among cross-types (Fig. 3). Marine fish from both locations had larger adductor and abductor muscles than sympatric stream-resident crosses. We did not find a significant effect of location on adductor (t_4=1.362, P=0.244) or abductor mass (t_4=1.41, P=0.2307).

We also examined pectoral muscles to determine whether there were metabolic differences in the muscles of stream-resident and marine fish. In general, a higher mitochondrial content should allow for higher use of oxygen in the muscle, and should facilitate a higher $\dot{M}_{O2,max}$. The mitochondrial content of muscles is tightly correlated with fibre type (reviewed by Zierath and Hawley, 2004), and can also be assessed by measuring the activity of muscle metabolic enzymes (e.g. Reichmann et al., 1985). We measured fibre-type composition in West Creek crosses (Fig. 4), and found that stickleback pectoral muscles were composed of three different fibre types, which we generally classified by fibre size and relative oxidative capacity (SDH staining intensity; Fig. 4). White fibres had a large cross sectional area (~3000–4000 μm^2) and were lightly stained for SDH, pink fibres were of intermediate size (~1200–2000 μm^2) and exhibited intermediate SDH staining, and red fibres were small (~600–1000 μm^2) and darkly stained for SDH (Fig. 4A, Tables 3, 4). The abductor muscle, which powers the recovery stroke, was composed of red, pink and white fibres (Fig. 4A). Stream-resident fish had a significantly lower percentage of red fibres in their pectoral abductor muscle than did marine fish, and hybrids had an intermediate percentage of red fibres ($F_{2,11}$=4.154, P=0.0453; Table 5). The adductor muscle, which powers the thrust-generating stroke, was composed of primarily red fibres (~600–1000 μm^2), but we also detected some fibres that were intermediate to red and pink fibres in size (~900–1800 μm^2) and staining, which we called 'red–pink' fibres (Fig. 4B–D). We found qualitative differences in the number of red–pink fibres (A.C.D., personal observations) and quantitative differences in their size (Table 4), such that stream-resident fish appeared to have more and larger red–pink fibres than marine fish.

We next investigated the metabolic capacity of pectoral muscles by measuring the activities of a suite of metabolic enzymes (CS, COX, PK, LDH and CPK; Table 6). These measures allowed us to compare our histological and enzymatic results in West Creek fish, so that we could gain insight into the metabolic characteristics of Bonsall Creek muscles (as we did not have histological samples from these crosses). Our histological data suggested that the adductor muscle is mainly composed of red fibres (high oxidative and low glycolytic capacity), but that the adductors of West Creek stream-resident fish also had a population of red–pink fibres (high oxidative and glycolytic capacity) that were not present in marine fish (Fig. 4, Table 4). Therefore, we predicted that stream-resident fish would have similar activities of mitochondrial enzymes (CS and COX) and higher activities of glycolytic enzymes (PK and LDH). As predicted, we found that West Creek stream-resident fish had similar activities of CS and COX, and higher LDH activities per gram adductor muscle (Table 6), but we did not detect differences in PK activity among ecotypes (Table 6). In general, our histological and enzymatic data for the pectoral adductor muscle were concordant.

Our histological data for the abductor muscles demonstrated that West Creek stream-resident fish had a lower percentage of red fibres than did West Creek marine fish (because of a combination of more white and pink fibres in stream-resident fish; Table 5). Thus, we

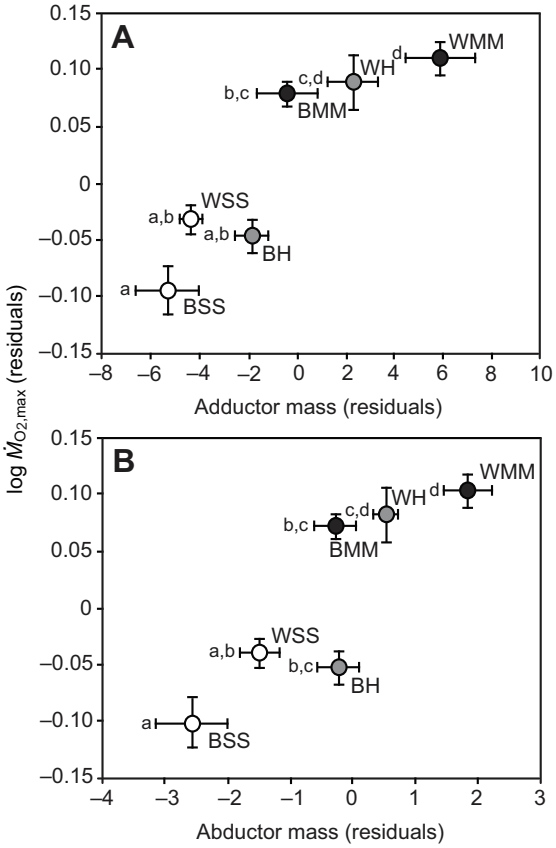

Fig. 3. Residual pectoral (A) adductor and (B) abductor mass of laboratory-bred F1 threespine stickleback females from Bonsall and West Creek parents plotted against residuals of log$\dot{M}_{O2,max}$. Abbreviations and data presentation follow Fig. 2. Bonsall Creek: *N*=5 SS, 11 H and 6 MM families; West Creek: *N*=5 SS, 5 H and 6 MM families.

predicted that West Creek stream-resident fish should have lower activities of aerobic enzymes (CS and COX), and higher activities of glycolytic enzymes (PK and LDH) and CPK in their abductor muscle. We did not find any significant differences in COX, CS, PK or CPK activities per gram of abductor muscle, but our predictions for LDH activities were met (Table 6). Overall, our histological and enzymatic data for West Creek abductor muscles did not match the predictions from the histological data perfectly, but both measures were able to detect fibre-type differences in the abductor and adductor muscle. Therefore, measuring LDH activities per gram adductor and abductor muscle in Bonsall Creek fish reflected differences in fibre-type composition.

Our combined enzymatic data for Bonsall and West Creek crosses displayed no significant differences among any cross-types in adductor COX and CS activity (COX, $F_{5,32}$=1.534, P=0.2069; CS, $F_{5,32}$=1.2841, P=0.2950), and no differences in adductor PK and CPK activity among ecotypes (PK, $F_{5,31}$=4.389, P=0.0039; CPK, $F_{5,31}$=3.7887, P=0.0086; Table 6). There were also no significant differences in COX ($F_{5,32}$=0.4954, P=7773) or CS ($F_{5,32}$=1.9332, P=0.1162) activities per gram of abductor muscle, and no ecotype-specific differences in CPK and PK activities per gram of abductor muscle (CPK, $F_{5,31}$=4.0223, P=0.0063; PK, $F_{5,32}$=4.9442, P=0.0018; Table 6). However, there were significant differences among ecotypes in LDH activity per gram of adductor ($F_{5,32}$=6.773, P=0.0002; Fig. 5A, Table 6) and abductor muscle ($F_{5,31}$=10.2467, P=0.0001; Fig. 5B, Table 6), such that marine crosses from both locations had significantly lower LDH activities than did stream-resident crosses from both locations (Table 6, Fig. 5A). The presence of similar activities of mitochondrial (CS and COX) and glycolytic (PK) enzymes, but different activities of LDH, suggests that stream-resident fish from both populations had a higher proportion of pink, fast-oxidative glycolytic fibres in their adductor and abductor muscles.

We also found some differences in enzyme activity between the pectoral adductor and abductor muscles. The adductor muscles from all cross-types had slightly higher COX, equivalent CS and CPK, and lower LDH and PK activities per gram than did the abductor muscles. These findings generally agree with our histological findings that the adductor (composed of red and some red–pink fibres) is more aerobic and less glycolytic than the abductor (composed of red, pink and white fibres). In addition, we studied the effect of sex on the size and enzyme activities of pectoral muscles. We found no effect on adductor or abductor size (see supplementary material Figs S2, S3), but we found significant differences in enzyme activities among sexes (supplementary material Table S1). Males had lower PK activities in their abductor and adductor muscles and slightly higher abductor and adductor COX, abductor CS and adductor CPK activies, and lower abductor LDH activities (supplementary material Table S1).

Our histological data also provided us with information on muscle fibre size (Tables 3, 4), which may influence the oxygen cascade by affecting the diffusion distance from the capillary to the mitochondria. We found that stream-resident fish from West Creek had significantly larger white ($F_{2,13}$=14.449, P<0.001) and pink fibres ($F_{2,13}$=5.752, P=0.0204; Table 3) in their abductor muscle. The size of red–pink fibres in West Creek stream-resident fish was also larger than these fibres in marine fish ($F_{2,13}$=5.942, P=0.0147).

Associations between $\dot{M}_{O2,max}$ and underlying traits related to oxygen transport and utilization

By measuring traits in F1 hybrids, we were also able to study the genetic architecture (i.e. additive *vs* non-additive genetic effects) of candidate traits and compare them with our findings for $\dot{M}_{O2,max}$ (Dalziel et al., 2012). If a given trait has a strong functional linkage to $\dot{M}_{O2,max}$, we expect it to evolve in the predicted direction and to have a genetic basis similar to that of $\dot{M}_{O2,max}$. Thus, we can reject the hypothesis of a strong functional linkage between a candidate trait and $\dot{M}_{O2,max}$ for traits that did not evolve as predicted after freshwater colonization, or that did not have a similar genetic architecture as $\dot{M}_{O2,max}$. We have previously shown that reductions in $\dot{M}_{O2,max}$ in Bonsall and West Creeks are the result of loci displaying strong dominance effects, such that stream alleles are dominant in Bonsall Creek F1 hybrids and marine alleles are dominant in West Creek F1 hybrids (Dalziel et al., 2012). Many traits which have evolved in stream-resident fish did not fit these patterns, such as fibre size (Tables 3, 4) in West Creek crosses, the percentage of red muscle in the abductor muscle (Table 5) of West Creek crosses, and abductor and adductor mass in Bonsall Creek crosses (Fig. 3). In West Creek crosses, abductor mass, adductor mass and the activity of LDH per gram of adductor and abductor muscle all displayed dominance effects similar to those of $\dot{M}_{O2,max}$. In Bonsall Creek, only ventricle mass and LDH per gram of abductor muscle displayed dominance effects similar to those of $\dot{M}_{O2,max}$. The only trait that displayed dominance effects similar to those of $\dot{M}_{O2,max}$ in both locations was the activity of LDH per gram of abductor muscle (based upon *post hoc* tests), but when we explicitly tested for differences in dominance among locations, we found a non-significant interaction (t_{31}=–1.965, P=0.0583). Note

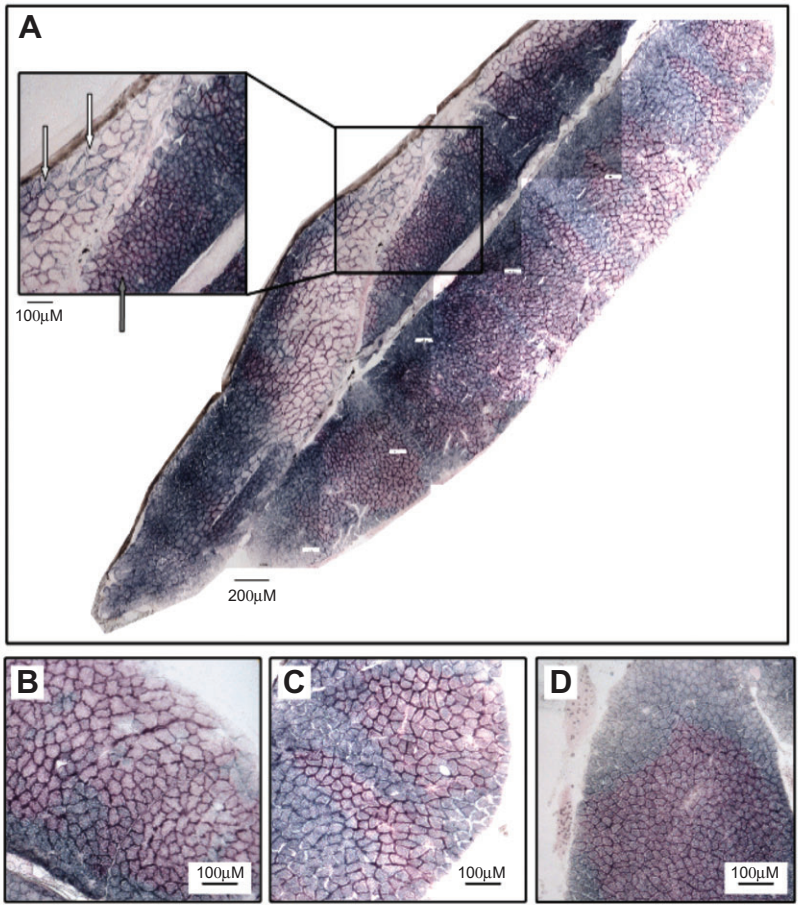

Fig. 4. Succinate dehydrogenase (SDH)-stained pectoral muscles from laboratory-bred F1 threespine stickleback females from West Creek parents. SDH, complex II in the mitochondrial electron transport chain, is used as a marker for oxidative capacity. (A) Representative section of the full pectoral muscle cut transverse to fibre length (produced from stitched images), with excerpt from the abductor muscle to display examples of red (dark grey arrow), pink (light grey arrow) and white (white arrow) fibres. Representative images of adductor muscles from (B) pure stream, (C) F1 hybrid and (D) pure marine fish.

that reciprocal F1 hybrid crosses did not vary in $\dot{M}_{O2,max}$ or any of the traits measured in this study (results not shown). These data suggest that mitochondrially encoded genes and/or parent-of-origin-dependent genomic imprinting do not significantly influence these traits.

DISCUSSION

Traits associated with reductions in $\dot{M}_{O2,max}$: stream-resident *vs* marine crosses

Differences in $\dot{M}_{O2,max}$ among individuals, populations or species could be due to variation in any of the sequential steps of the oxygen cascade: (1) convection of oxygen across the respiratory surface, (2) diffusion from the respiratory surface to the blood, (3) convection through the blood, (4) diffusion from the capillary to the mitochondria or (5) use of oxygen in the electron transport chain (reviewed by Taylor and Weibel, 1981; Wagner, 1996). We examined a series of traits in the oxygen cascade to determine whether they evolved in conjunction with decreases in $\dot{M}_{O2,max}$. The theory of symmorphosis predicts that all steps in the oxygen cascade will have evolved to match the lowered $\dot{M}_{O2,max}$ in stream-resident fish, because maintaining an excess capacity in any of these traits is energetically costly. Evolutionary theory predicts that the traits which are the most costly to maintain, either for energetic reasons or because of functional trade-offs with other performance traits (Lahti et al., 2009), should be the first to evolve after stream-resident fish evolved a lower $\dot{M}_{O2,max}$. These two hypotheses both argue that maintenance costs of 'excess capacity' should drive evolutionary change. However, evolutionary theory does not predict that all steps in the oxygen cascade will have evolved together, and also makes predictions about the order of evolutionary change (e.g. which traits will evolve first). We have used mechanistic knowledge about possible trade-offs with other performance traits

Table 3. Fibre area (and residuals) for red, pink and white fibres from abductor muscles of laboratory-bred F1 threespine stickleback females from West Creek parents

	Red fibre area (μm^2)	Residual red fibre area	Pink fibre area (μm^2)	Residual pink fibre area	White fibre area (μm^2)	Residual white fibre area
Stream (*N*=4 families)	780.3±92.6	35.0±66.8	1950.9±250.4	204.7±93.4[a]	4228.1±489.6	758.1±177.2[a]
Hybrid (*N*=6 families)	955.6±109.8	194.5±100.5	2085.6±210.7	311.4±188.0[a]	3674.8±149.1	125.7±186.5[a]
Marine (*N*=5 families)	640.7±88.7	–82.0±72.9	1400.3±106.2	–293.2±97.8[b]	2667.2±224.2	–690.4±218.3[b]

Data are presented as the grand means ± s.e.m. of all family means; but statistical tests included all individuals in a nested ANOVA model (see Results). There was a significant effect of mass on all values, so residuals were used for statistical tests, but raw data are also presented here for clarity. Different superscripted letters indicate significant differences among groups for a given measure (P<0.05). If letters are not included, there were no significant differences among the three groups.

Table 4. Fibre area (and residuals) for the largest and smallest red fibres in the adductor muscles of laboratory-bred F1 threespine stickleback females from West Creek parents

	Smallest red fibre area (μm^2)	Red fibre area residuals	Largest red–pink fibre area (μm^2)	Red–pink fibre area residuals
Stream (*N*=4 families)	972.5±199.2	130.8±180.4	1821.4±189.5[a]	347.1±190.9[a]
Hybrid (*N*=6 families)	1043.8±104.4	185.4±91.9	1800.7±322.1[a]	284.4±302.5[a]
Marine (*N*=5 families)	667.3±39.2	–150.7±39.2	904.6±59.9[b]	–529.5±42.7[b]

Data are presented as the grand means ± s.e.m. of all family means, but statistical tests included all individuals in a nested ANOVA model (see Results). There was a significant effect of mass on all values, so residuals were used for statistical tests, but raw data are also presented here for clarity. Different superscripted letters indicate significant differences among groups for a given measure (P<0.05). If letters are not included, there were no significant differences among the three groups.

[e.g. the osmo-respiratory compromise influencing fish gill size (Sardella and Brauner, 2007) and muscle fibre size (Johnston et al., 2005; Jimenez et al., 2011)] and the energetic costs of trait maintenance [e.g. the cost of maintaining large cardiac and skeletal muscle masses (e.g. Daan et al., 1990; Zera et al., 1998)] to form a set of predictions as to which oxygen cascade traits may have evolved in stream-resident fish in the relatively short (<12,000 years) time since they diverged from marine sticklebacks. Specifically, we predicted that stream-resident populations with a lower $\dot{M}_{O2,max}$ would have evolved smaller gill surface areas, lower Hct, smaller hearts, smaller pectoral muscles and larger pectoral muscle fibres than their migratory marine ancestors.

Contrary to our predictions, we found no differences in gill surface area among stream-resident and marine ecotypes reared in a common laboratory environment at a salinity of 2 p.p.t. (Table 1). This was surprising, because in addition to the potential impact on $\dot{M}_{O2,max}$, we predicted that a smaller gill surface area would experience positive selection in freshwater in sticklebacks. This is because the fish gill is the primary site of osmoregulation and respiration, causing a trade-off between these two tasks (reviewed by Sardella and Brauner, 2007), and after colonization of freshwater, a new source of selection was applied in sticklebacks: selection for survival in freshwater streams and lakes during the cold winter months. Ancestral marine sticklebacks cannot successfully osmoregulate under these conditions, and experience high mortality rates in freshwater at cold temperatures (Schaarschmidt et al., 1999). Thus, it has been hypothesized that stream-resident fish experienced strong selection for survival in the cold, low-salinity waters, which they now inhabit (e.g. Heuts, 1945; Guderley, 1994; Schaarschmidt et al., 1999). Indeed, lake-resident stickleback populations can survive at lower temperatures than marine sticklebacks in freshwater (Barrett et al., 2011).

These ecological changes suggest that smaller gills, which are predicted to be favoured during osmoregulatory challenges, would be selected for in freshwater. It is possible that other traits related to oxygen uptake and ion leakage at the gill (which we did not measure in the present study), such as lamellar blood-to-water diffusion distance, gill perfusion or ventilation rate, may have evolved in threespine sticklebacks after freshwater colonization (e.g. Henriksson et al., 2008). Fish gills are also highly plastic, and are able to remodel when exposed to changes in salinity, oxygen concentrations and temperature (reviewed by Nilsson, 2007), so it is also possible that any differences in gill surface area among ecotypes would only be observed after acclimation to cold freshwater. Our predictions for gill surface area are also complicated by the differences in growth rate found among low-plated (stream-resident phenotype) and fully-plated (marine phenotype) sticklebacks, such that low-plated fish have a faster growth rate in freshwater (Marchinko and Schluter, 2007; Barrett et al., 2009). Thus, it is possible that a high capacity for oxygen uptake is needed to maintain the high growth rates of freshwater ecotypes, so that smaller gill surface area is not favoured in these populations. Finally, it is possible that evolutionary constraints [e.g. a lack of genetic variation (reviewed by Garland and Carter, 1994; Feder et al., 2000; Brakefield and Roskam, 2006; Futuyma, 2010)] have limited the evolution of gill surface area and related physiological traits.

Decreases in Hct and MCHC could also contribute to reductions in $\dot{M}_{O2,max}$ by reducing the oxygen carrying capacity of the blood. Increases in Hct increase blood viscosity (e.g. Egginton, 1996) and the energetic costs of pumping blood. However, these costs may not be significant at the Hct levels and temperatures common for sticklebacks (Gallaugher et al., 1995). We did not find any differences in Hct or MCHC among stream-resident and marine crosses (Table 2). We also measured the whole-blood-cell hemoglobin–oxygen binding affinity (Hb P_{50}) of wild Bonsall Creek stream-resident and marine fish, and did not find significant differences among ecotypes (see supplementary material Fig. S1). Together, these data suggest that differences in the oxygen carrying capacity of the blood have not evolved in conjunction with decreases in $\dot{M}_{O2,max}$ in stream-resident sticklebacks.

Reductions in cardiac output can also decrease $\dot{M}_{O2,max}$ *via* decreases in oxygen convection. Indeed, cardiac output is correlated with $\dot{M}_{O2,max}$ in salmonids (e.g. Claireaux et al., 2005; Eliason et al., 2011), and if all else is equal, ventricle size should correlate with cardiac output. Traits that are costly to maintain may undergo particularly rapid evolution for trait loss when selection is relaxed (Lahti et al., 2009). Thus, ventricle mass might be predicted to

Table 5. Percentage of each fibre type (and residuals) in the abductor muscles of laboratory-bred F1 females from West Creek parents

	% Red fibres	Residual % red fibres	% Pink fibres	Residual % pink fibres	% White fibres	Residual % white fibres
West stream (*N*=4 families)	64.17±3.48	–6.85±3.28[a]	11.60±1.50	0.82±1.65	24.23±4.59	6.03±4.60
West hybrid (*N*=5 families)	71.49±3.11	0.13±3.33[a,b]	9.83±1.18	–1.06±1.15	18.69±2.58	0.93±2.88
West marine (*N*=5 families)	77.18±2.42	5.46±2.61[b]	9.47±1.82	–1.54±1.71	13.35±1.14	–3.92±0.99

Data are presented as the grand means ± s.e.m. of all family means, but statistical tests included all individuals in a nested ANOVA model (see Results). There was a significant effect of mass on all values, so residuals were used for statistical tests, but raw data are also presented here for clarity. Different superscripted letters indicate significant differences among groups for a given measure (P<0.05). If letters are not included, there were no significant differences among the three groups.

Table 6. Enzyme activities of laboratory-bred F1 threespine stickleback females from Bonsall and West Creek parents

	Enzyme activity (U enzyme g^{-1})		
	Ventricle	Adductor	Abductor
Cytochrome *c* oxidase (COX)			
Bonsall stream	39.01±3.14^{c}	34.52±5.23	31.15±5.81
West stream	62.20±7.49^{a,b}	36.42±5.08	34.41±4.47
Bonsall hybrid	47.17±3.79^{c}	34.87±2.67	32.15±2.07
West hybrid	87.00±10.1^{a}	40.38±5.65	34.42±2.84
Bonsall marine	51.00±3.8^{b,c}	36.62±3.03	30.23±5.07
West marine	47.06±6.3^{b,c}	44.46±4.04	32.99±4.25
Citrate synthase (CS)			
Bonsall stream	11.20±0.76	11.38±1.41	10.87±0.80
West stream	14.95±1.29	13.81±1.71	13.70±0.87
Bonsall hybrid	13.41±1.04	11.45±0.86	11.67±0.74
West hybrid	15.98±1.40	13.86±2.17	13.20±1.29
Bonsall marine	14.45±0.72	12.11±0.87	12.06±0.71
West marine	12.46±1.36	12.94±0.97	11.01±0.88
Pyruvate kinase (PK)			
Bonsall stream	47.43±1.58	73.93±7.91^{a,b,c}	76.94±10.10^{c,d}
West stream	42.66±4.60	88.61±8.73^{a}	112.54±11.70^{a}
Bonsall hybrid	43.12±2.55	73.09±5.08^{b,c}	83.83±6.28^{b,c,d}
West hybrid	48.78±3.53	88.45±4.02^{a}	104.51±6.45^{a,b}
Bonsall marine	44.93±1.35	53.52±4.50^{c}	65.34±5.61^{d}
West marine	45.03±2.73	83.33±6.87^{a,b}	98.87±8.70^{a,b,c}
Lactate dehydrogenase (LDH)			
Bonsall stream	39.73±5.67	22.26±1.85^{a}	31.61±0.76^{a,b}
West stream	44.94±4.02	18.05±2.23^{a,b}	36.45±3.20^{a}
Bonsall hybrid	49.36±2.86	14.39±2.17^{a,b,c}	28.62±2.08^{a,b,c}
West hybrid	44.00±5.91	9.94±1.40^{b,c,d}	22.32±3.24^{b,c,d}
Bonsall marine	43.58±6.01	6.65±1.17^{d}	14.58±3.59^{d}
West marine	31.61±2.12	4.94±1.13^{d}	13.70±2.11^{d}
Creatine phosphokinase (CPK)			
Bonsall stream	298.22±48.07	589.05±71.37^{a,b}	647.67±60.06^{a,b}
West stream	202.45±23.71	797.67±39.86^{a}	846.09±25.72^{a}
Bonsall hybrid	296.58±20.32	755.61±27.40^{a}	703.21±55.71^{a}
West hybrid	281.59±30.85	749.36±37.17^{a}	692.96±53.05^{a}
Bonsall marine	355.59±23.75	593.84±62.84^{b}	471.81±37.27^{b}
West marine	309.47±47.90	723.57±44.16^{a,b}	621.79±82.65^{a,b}

Data are presented as the grand means ± s.e.m. of all family means, but statistical tests included all individuals in a nested ANOVA model (see Results). The number of families used to calculate enzyme activities is as follows: *N*=5 Bonsall Creek SS, 11 H and 6 MM families and 5 West Creek SS, 5 H, and 6 MM families for ventricle enzyme activities. *N*=4 Bonsall Creek SS, 11 H and 6 MM families and 5 West Creek SS, 5 H, and 6 MM families for adductor and abductor enzyme activities. Different superscripted letters indicate significant differences among groups for the indicated enzyme within a single tissue (P<0.05). If letters are not included, there were no significant differences in enzyme activity within the tissue among the six groups.

evolve rapidly if the energetic costs of maintaining a large heart are high. Indeed, heart size is positively associated with basal metabolic rate among species of birds (e.g. Daan et al., 1990) and strains of laboratory mice (e.g. Konarzewski and Diamond, 1995; Brzęk et al., 2007), suggesting high maintenance costs. However, heart mass is also correlated with the mass of other metabolically active organs (Daan et al., 1990; Konarzewski and Diamond, 1995; Brzęk et al., 2007), and is not always correlated with basal metabolic rate (e.g. Chappell et al., 2007), suggesting that the metabolic costs of maintaining a large heart are context dependent. The differences in growth rate among stickleback ecotypes also complicate our predictions for ventricle mass evolution, as a high capacity for convective oxygen transport may also be needed to maintain high growth rates. We found variation in ventricle size among ecotypes, but these effects differed among locations: Bonsall Creek stream-resident crosses had significantly smaller hearts than sympatric marine crosses, but West Creek stream-resident crosses had ventricles that did not differ significantly from those of sympatric marine crosses (Fig. 2). However, we found no differences between marine and stream-resident crosses in the activity per gram of ventricle of CS or COX (Table 6), suggesting that there have been no evolutionary changes in cardiac aerobic capacity after freshwater colonization. These findings suggest that ventricle size may contribute to decreases in $\dot{M}_{O2,max}$ in Bonsall Creek but not West Creek stream-resident fish, and also argue that having a ventricle as large as that of a Bonsall Creek marine fish is not necessary for reaching a high $\dot{M}_{O2,max}$.

After oxygen is transported to the working muscle, it must diffuse from the capillary to the muscle mitochondria. If all else is equal, smaller fibres will decrease the diffusion distance to the mitochondria compared with larger fibres (Kinsey et al., 2011), but incur higher costs from maintaining ion gradients, resulting in an energetic trade-off [‘optimal fibre number hypothesis’ (e.g. Johnston et al., 2005; Jimenez et al., 2011)]. We predicted that if selection for a high oxygen diffusion rate were relaxed, stream-resident fish with larger fibres would have a decreased cost of maintaining ion gradients and be at a selective advantage. In agreement with these predictions, we found that stream-resident fish from West Creek had larger pink and white fibres in their abductor muscles (Table 3), and had larger red–pink fibres in their adductor muscles (Table 4). There was also a non-significant trend towards larger red abductor and adductor fibres in stream-resident

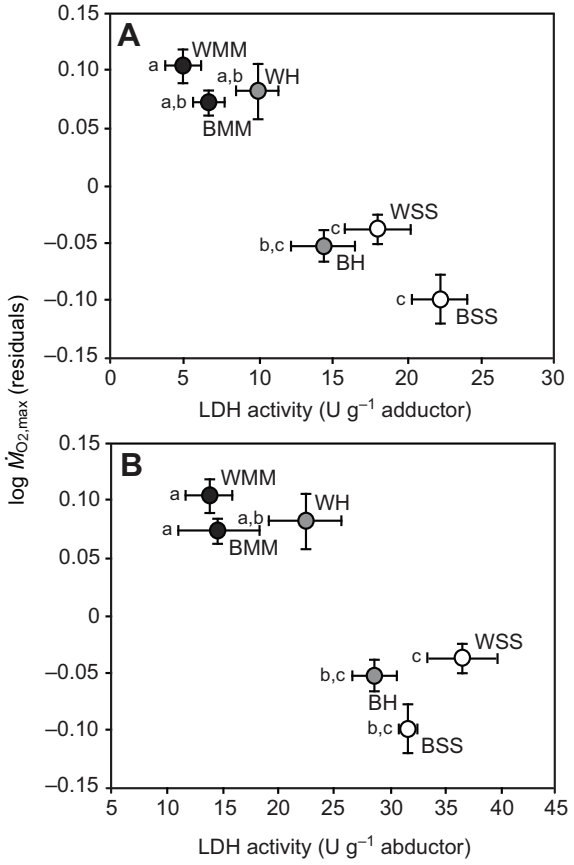

Fig. 5. Lactate dehydrogenase (LDH) activity per gram of (A) adductor and (B) abductor muscle from laboratory-bred F1 threespine stickleback females from Bonsall and West Creek parents plotted against residuals of log$\dot{M}_{O2,max}$. Abbreviations and data presentation follow Fig. 2. Bonsall Creek: N=5 SS, 11 H and 6 MM families; West Creek: N=5 SS, 5 H and 6 MM families.

fish (Tables 3, 4). Further studies examining the ion transporter content of stickleback muscles are needed to determine whether changes in muscle size impact the costs of ion regulation as they do in lobster muscle fibres (Jimenez et al., 2011), and measurements of mitochondrial placement within a fibre, myoglobin content and muscle capillarity are needed to examine other possible differences in oxygen diffusion capacity (e.g. Scott et al., 2009; Kinsey et al., 2011).

The final step in the oxygen transport cascade involves the reduction of oxygen by COX in the mitochondrial electron transport chain, and the size and mitochondrial content of the working muscles generally correlates with $\dot{M}_{O2,max}$ (reviewed by Wagner, 1996; Hoppeler and Wiebel, 1998). In labriform swimmers such as threespine stickleback, prolonged swimming is powered with the pectoral muscles (Taylor and McPhail, 1986; Walker, 2004), with the pectoral adductor powering the forward thrust and the pectoral abductor powering the recovery stroke (Thorsen and Weastneat, 2005). If the energetic costs of maintaining large pectoral muscles are high, then we would predict that stream-resident fish, which have a lower $\dot{M}_{O2,max}$ than marine stickleback populations (Dalziel et al., 2012), would evolve smaller pectoral muscles with a lower aerobic capacity per gram muscle. Indeed, large, metabolically active muscles are energetically expensive for sand crickets to maintain (e.g. Zera et al., 1998): sand crickets (genus *Gryllus*) that develop into a long-winged form capable of flight have larger flight muscles, which oxidize more lipids, and have higher muscle metabolic rates, higher whole-animal standard and maximum metabolic rates, and smaller gonads than short-winged forms with smaller muscles (Zera et al., 1997; Zera et al., 1998; Crnokrak and Roff, 2002; Nespolo et al., 2008). As predicted, we found that pectoral muscle size was reduced in both stream-resident populations (Fig. 3). To assess the aerobic capacity of stickleback pectoral muscle, we measured two traits that are correlated with mitochondrial content: (1) fibre-type composition (Zierath and Hawley, 2004), and (2) the activities of two mitochondrial enzymes, CS and COX (e.g. Reichmann et al., 1985). We found that West Creek stream-resident fish had a lower percentage of red fibres in their abductor muscle (Table 5) and more pink fibres in their adductor muscle (Fig. 4, Table 4) than West Creek marine fish (Bonsall Creek crosses were not studied by this method), but we did not find evidence for differences in CS and COX activities per gram tissue (Table 6). These data suggest that stream-resident fish have evolved a higher percentage of pink fibres, which have high glycolytic and aerobic capacites, and so do not reduce the capacity for oxygen utilization in the pectoral muscle. Overall, these data indicate that differences in pectoral muscle size, but not mitochondrial content per gram pectoral muscle, are associated with a decreased $\dot{M}_{O2,max}$ in stream-resident stickleback. We hypothesize that the energetic costs of maintaining large pectoral muscles, with small-diameter fibres, may have resulted in selection for parallel reductions in pectoral muscle mass in stream-resident threespine stickleback populations.

In summary, reductions in the aerobic capacity of stream-resident stickleback are associated with reductions in a number of candidate oxygen-cascade-related traits, including a reduced pectoral muscle size in Bonsall and West Creek stream-resident fish, differences in pectoral muscle fibre size in West Creek stream-resident fish (not measured in Bonsall Creek crosses), and decreases in ventricle size in Bonsall Creek stream-resident fish. Previous studies on wild stream-resident and marine sticklebacks from another population in the Fraser river, BC, Canada (Salmon River), have identified similar decreases in pectoral muscle mass and heart mass (Hochackha and Somero, 2002) (C. A. Darveau and P. W. Hochachka, unpublished data), and wild stream-resident fish from the Baltic Sea tributaries also have smaller pectoral muscles than sympatric marine fish (Schaarschmidt and Jurss, 2003). Overall, these data, in combination with our results, suggest that decreases in pectoral muscle size have evolved in parallel with decreases in $\dot{M}_{O2,max}$ in both Pacific and Atlantic stream-resident threespine stickleback populations. We also found evidence for the evolution of fibre-type composition, but not mitochondrial content (i.e. activities of CS and COX per gram muscle), in the pectoral muscles of stream-resident fish. Changes in pectoral fibre type that do not result in differences in mitochondrial content (measured using CS and COX as proxies) should not impact $\dot{M}_{O2,max}$ directly, but may impact swimming performance (e.g. Dalziel et al., 2012). Previous studies (Hochachka and Somero, 2002; Schaarschmidt and Jurss, 2003) (C. A. Darveau and P. W. Hochachka, unpublished data) have found similar differences in LDH activities per gram muscle in wild-caught stream-resident *vs* marine stickleback. Overall, these independent changes in $\dot{M}_{O2,max}$, pectoral muscle size and fibre type (indicated by LDH activity), which occurred in less than 12,000 years in the face of gene flow from marine populations (e.g. Hagen, 1967; Jones et al., 2006) (T. H. Vines and A.C.D., unpublished data), are consistent with a role for natural selection in the evolution of these physiological traits in stream-resident stickleback populations.

Comparisons among marine populations

Local adaptation to migratory conditions may occur in anadromous fish that display natal philopatry. For example, Eliason et al. (Eliason et al., 2011) found that the cardiac physiology of sockeye salmon in the Fraser River, BC, Canada, is correlated with migratory difficulty. Marine populations of threespine stickleback also display some degree of homing (Saimoto, 1993), and are genetically differentiated [although much less so than freshwater populations (Withler and McPhail, 1985; Colosimo et al., 2005)], so there is the potential for local adaptation to migratory conditions in this species as well.

Our two marine populations vary in the difficulty of their anadromous migrations: West Creek marine fish travel at least 35 km down the Fraser River to reach the ocean, but Bonsall Creek stickleback breed only 1–2 km from the mouth of the estuary (Hagen, 1967) (T. H. Vines and A.C.D., unpublished data). We found that multiple traits related to oxygen transport and utilization, muscle metabolic capacity and swimming performance were higher in West Creek marine crosses than in Bonsall Creek marine crosses, including Hct values in female fish (Table 2), pectoral muscle mass (Fig. 3), pectoral muscle PK activity (Table 6) and body streamlining (Dalziel et al., 2012). However, Bonsall Creek marine fish had larger ventricles than West Creek marine fish. These data suggest that marine populations do form genetically distinct populations and, with the exception of ventricle mass, their phenotypes are generally consistent with our predictions for local adaptation to migratory conditions. In addition, the combination of a large ventricle (high capacity for blood convection), but a smaller pectoral muscle mass (lower capacity for oxygen use) in Bonsall Creek fish, and small ventricles (lower capacity for blood convection) and larger pectoral muscles (higher capacity for oxygen use) in West Creek fish, further argues against a tight matching of the capacities of all steps in the oxygen transport cascade at any given point in evolutionary time (i.e. symmorphosis). Overall, marine populations are able to reach similar maximum metabolic rates (Fig. 2) (Dalziel et al., 2012), but likely use different underlying mechanisms to reach high performance values, a phenomenon called many-to-one mapping (reviewed by Wainwright et al., 2005; Walker, 2010).

If ancestral marine populations also displayed physiological variation at the time of freshwater colonization, any current differences among freshwater populations may also be due to differences in the genetic 'starting point' at the time of colonization. It is possible that the relatively limited number of families from each population (five to 11 crosses of each cross-type), might not express the full amount of variation present in these natural populations. However, the fact that we observed cross-types with significantly different $\dot{M}_{O_2,max}$ values and significant differences in which oxygen-cascade traits associate with a given capacity for $\dot{M}_{O_2,max}$ clearly argues that a high $\dot{M}_{O_2,max}$ is not always reached *via* the same mechanism.

Genetic basis of $\dot{M}_{O_2,max}$ and underlying traits

If an underlying trait has had a major effect on $\dot{M}_{O_2,max}$, then we would expect that the loci contributing to variation in this trait will also account for a large amount of the variation in $\dot{M}_{O_2,max}$. Thus, any traits that are strongly mechanistically related to $\dot{M}_{O_2,max}$ should have a genetic basis similar to that of $\dot{M}_{O_2,max}$, which we have found to show dominance of marine alleles in West Creek F1 hybrids and a dominance of stream alleles in Bonsall Creek F1 hybrids. Although there were parallel decreases in pectoral muscle size in both stream-resident populations, the traits that had a genetic basis similar to that of $\dot{M}_{O_2,max}$ differed among locations. In West Creek, abductor and adductor masses matched the genetic architecture of $\dot{M}_{O_2,max}$ (i.e. dominance of marine alleles) and in Bonsall Creek, ventricle mass matched the genetic architecture of $\dot{M}_{O_2,max}$ (i.e. dominance of stream alleles). Thus, none of our oxygen-cascade-related traits had a genetic basis similar to that of $\dot{M}_{O_2,max}$ in both locations. These data argue that although there are some oxygen-cascade-related traits that have repeatedly evolved in stream-resident fish (reductions in pectoral muscle size), the particular traits predicted to have a large impact on $\dot{M}_{O_2,max}$ (e.g. those that share a similar genetic basis) differ among populations. These findings argue that reductions in $\dot{M}_{O_2,max}$ may also happen in a variety of different ways (e.g. many-to-one mapping).

Does the stickleback oxygen cascade demonstrate symmorphosis?

The hypothesis of symmorphosis argues that there should be a 'quantitative match of design and function parameters within a defined functional system' (Weibel et al., 1991). Thus, if the stickleback respiratory system evolved in a symmorphotic manner, we would expect changes in $\dot{M}_{O_2,max}$ to be accompanied by changes in all steps of the oxygen cascade from the gill to the mitochondria within the swimming muscles. Overall, our data strongly argue against a tight matching of capacity at all steps of the oxygen cascade in threespine stickleback. Our first example comes from our comparisons of stream-resident and marine ecotypes. Despite large differences in $\dot{M}_{O_2,max}$ between these ecotypes (Dalziel et al., 2012), a number of steps in the oxygen cascade did not differ, including gill surface area (Table 1), MCHC and Hct (Table 2), Hb P_{50} (see supplementary material Fig. S1) and the activities of mitochondrial enzymes per gram of pectoral muscle (CS and COX; Table 6). Our next example is from our comparisons of marine populations, which can reach a similar $\dot{M}_{O_2,max}$, but vary in a number of oxygen-cascade-related traits [e.g. pectoral muscle size (Fig. 3), ventricle size (Fig. 2) and Hct (Table 2)]. In particular, Bonsall Creek marine fish show evidence of a high capacity for blood convection (i.e. large ventricle), but a lower capacity for oxygen use (i.e. a smaller pectoral muscle mass), whereas West Creek fish show evidence of a lower capacity for blood convection (i.e. small ventricles), but a higher capacity for oxygen use (i.e. larger pectoral muscles). Together, these comparisons argue against a tight matching of the capacities of all steps in the oxygen transport cascade at any given point in evolutionary time.

Physiological systems often influence more than one ecologically important task. This 'multi-tasking' can lead to trade-offs among performance traits, and is one of the major reasons why perfectly matched systems (as predicted under symmorphosis) are unlikely to evolve in natural populations (Weibel et al., 1991; Garland, 1998). For example, because at least one of the traits involved in the fish respiratory system also has another dominant function and is expected to experience trade-offs among functions (e.g. the gill is the primary site of both osmoregulation and respiration), we would predict that the respiratory system in fish is less likely, compared with the respiratory system of mammals, to display strict symmorphosis (Weibel et al., 1991). However, even in the mammalian respiratory system there is evidence for mismatches between structure (i.e. lung capacity) and function (i.e. $\dot{M}_{O_2,max}$) (Weibel et al., 1991). Garland (Garland, 1998) has reviewed a number of further reasons why symmorphosis is unlikely to occur in nature, which include, but are not limited to: limitations to biological materials, limitations to selection, the importance of sexual selection and the importance of stochastic evolutionary processes. So why is it common to find evidence for the matching of design and function in interspecific

comparisons? We argue that this observation is simply a result of the different time scales available for divergence between interspecific and intraspecific comparisons. Interspecific studies observe the many changes that have occurred over longer evolutionary time scales, whereas intraspecific studies are more likely to detect the first few 'steps' of evolutionary change. This point is best exemplified by selection studies in rodents, which clearly display that there has been very rapid, step-wise evolution of aerobic capacity, which would have been missed if it was not studied every few generations (e.g. Henderson et al., 2002; Howlett et al., 2009; Kirkton et al., 2009; Gębczyński and Konarzewski, 2011).

LIST OF SYMBOLS AND ABBREVIATIONS

CPK	creatine phosphokinase
COX	cytochrome *c* oxidase
CS	citrate synthase
H	hybrid cross
[Hb]	hemoglobin concentration
Hb P_{50}	whole-blood-cell hemoglobin–oxygen binding affinity
Hct	hematocrit
LDH	lactate dehydrogenase
MCHC	mean cellular hemoglobin content
$\dot{M}_{O_2,max}$	maximum oxygen consumption
MM	marine × marine cross
MS	marine × stream resident cross
PK	pyruvate kinase
SDH	succinate dehydrogenase
SM	stream resident × marine cross
SS	stream resident × stream resident cross

ACKNOWLEDGEMENTS

We thank C. A. Darveau for inspiring us to do this research, sharing unpublished data and providing experimental advice. We are grateful to D. Schluter for sharing his knowledge of stickleback natural history, providing statistical advice,and sharing his stickleback rearing facilities. We thank G. R. Scott for many helpful discussions and technical assistance with muscle fibre-type measurements, and R. S. Dhillon for his help with histological sampling and assays. We also thank A. Fong, C. Reyes and C. Porteus for their assistance with microscopy and histological preparations; B. Milsom for use of his cryostat; J. G. Richards for assistance in the measurement of gill morphology; and D. S. Srivastava for use of her microscope.

FUNDING

This work was supported by the Natural Sciences and Engineering Research Council of Canada through Discovery and Discovery Accelerator Grants to P.M.S. and a Canada Graduate Scholarship to A.C.D.

REFERENCES

Barrett, R. D. H., Rogers, S. M. and Schluter, D. (2009). Environment specific pleiotropy facilitates divergence at the ectodysplasin locus in threespine stickleback. *Evolution* **63**, 2831-2837.

Barrett, R. D. H., Paccard, A., Healy, T. M., Bergek, S., Schulte, P. M., Schluter, D. and Rogers, S. M. (2011). Rapid evolution of cold tolerance in stickleback. *Proc. R. Soc. Lond. B* **278**, 233-238.

Bell, M. A. and Foster, S. A. (eds) (1994). *The Evolutionary Biology of the Threespine Stickleback*. Oxford, UK: Oxford University Press.

Bennett, A. F. (1989). Integrated studies of locomotor performance. In *Complex Organismal Functions: Integration and Evolution in Vertebrates* (ed. D. B. Wake and G. Roth), pp. 191-202. Chichester: John Wiley and Sons.

Bennett, A. F. (1991). The evolution of activity capacity. *J. Exp. Biol.* **160**, 1-23.

Bernal, D., Dickson, K. A., Shadwick, R. E. and Graham, J. B. (2001). Analysis of the evolutionary convergence for high performance swimming in lamnid sharks and tunas. *Comp. Biochem. Physiol.* **129A**, 695-726.

Blaxhall, P. C. and Daisley, K. W. (1973). Routine haematological methods for use with fish blood. *J. Fish. Biol.* **5**, 771-781.

Brakefield, P. M. and Roskam, J. C. (2006). Exploring evolutionary constraints is a task for an integrative evolutionary biology. *Am. Nat.* **168**, S4-S13.

Brzęk, P., Bielawska, K., Książek, A. and Konarzewski, M. (2007). Anatomic and molecular correlates of divergent selection for basal metabolic rate in laboratory mice. *Physiol. Biochem. Zool.* **80**, 491-499.

Chappell, M. A., Garland, T., Robertson, G. F. and Saltzman, W. (2007). Relationships among running performance, aerobic physiology and organ mass in male Mongolian gerbils. *J. Exp. Biol.* **210**, 4179-4197.

Claireaux, G., McKenzie, D. J., Genge, A. G., Chatelier, A., Aubin, J. and Farrell, A. P. (2005). Linking swimming performance, cardiac pumping ability and cardiac anatomy in rainbow trout. *J. Exp. Biol.* **208**, 1775-1784.

Colosimo, P. F., Hosemann, K. E., Balabhadra, S., Villarreal, G., Dickson, M., Grimwood, J., Schmutz, J., Myers, R. M., Schluter, D. and Kingsley, D. M. (2005). Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science* **307**, 1928-1933.

Crnokrak, P. and Roff, D. A. (2002). Trade-offs to flight capability in *Gryllus firmus*: the influence of whole-organism respiration rate on fitness. *J. Evol. Biol.* **15**, 388-398.

Daan, S., Masman, D. and Groenewold, A. (1990). Avian basal metabolic rates: their association with body composition and energy expenditure in nature. *Am. J. Physiol.* **259**, R333-R340.

Dalziel, A. C., Rogers, S. M. and Schulte, P. M. (2009). Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. *Mol. Ecol.* **18**, 4997-5017.

Dalziel, A. C., Vines, T. H. and Schulte, P. M. (2012). Reductions in prolonged swimming capacity following freshwater colonization in multiple threespine stickleback populations. *Evolution* (in press).

Dhillon, R. S. and Schulte, P. M. (2011). Intra-specific variation in the thermal plasticity of mitochondria in killifish. *J. Exp. Biol.* **214**, 3639-3648.

Egginton, S. (1996). Blood rheology of Antarctic fishes: viscosity adaptations at very low temperatures. *J. Fish Biol.* **48**, 513-521.

Eliason, E. J., Clark, T. D., Hague, M. J., Hanson, L. M., Gallagher, Z. S., Jeffries, K. M., Gale, M. K., Patterson, D. A., Hinch, S. G. and Farrell, A. P. (2011). Differences in thermal tolerance among sockeye salmon populations. *Science.* **332**, 109-112.

Farrell, A. P., Johansen, J. A. and Suarez, R. K. (1991). Effects of exercise-training on cardiac performance and muscle enzymes in rainbow trout, *Oncorhynchus mykiss*. *Fish. Physiol. Biochem.* **9**, 303-312.

Feder, M. E., Bennett, A. F. and Huey, R. B. (2000). Evolutionary physiology. *Annu. Rev. Ecol. Syst.* **31**, 315-341.

Frappell, P., Schultz, T. and Christian, K. (2002). Oxygen transfer during aerobic exercise in a varanid lizard *Varanus mertensi* is limited by the circulation. *J. Exp. Biol.* **205**, 2725-2736.

Futuyma, D. J. (2010). Evolutionary constraint and ecological consequences. *Evolution* **64**, 1865-1884.

Gallaugher, P., Thorarensen, H. and Farrell, A. P. (1995). Hematocrit in oxygen transport and swimming in rainbow trout (*Oncorhynchus mykiss*). *Respir. Physiol.* **102**, 279-292.

Garland, T., Jr (1998). Testing the predictions of symmorphosis: conceptual and methodological issues. In *Principles of Animal Design: The Optimization and Symmorphosis Debate* (ed. E. R. Weibel, L. Bolis and C. R. Taylor), pp. 40-47. Cambridge, UK: Cambridge University Press.

Garland, T., Jr and Bennett, A. F. (1990). Quantitative genetics of maximal oxygen consumption in a garter snake. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **259**, R986-R992.

Garland, T., Jr and Carter, P. A. (1994). Evolutionary physiology. *Annu. Rev. Physiol.* **56**, 579-621.

Gębczyński, A. K. and Konarzewski, M. (2011). Effects of oxygen availability on maximum aerobic performance in *Mus musculus* selected for basal metabolic rate or aerobic capacity. *J. Exp. Biol.* **214**, 1714-1720.

Gonzalez, N. C., Kirkton, S. D., Howlett, R. A., Britton, S. L., Koch, L. G., Wagner, H. E. and Wagner, P. D. (2006). Continued divergence in $\dot{V}_{O_2\,max}$ of rats artificially selected for running endurance is mediated by greater convective blood O_2 delivery. *J. Appl. Physiol.* **101**, 1288-1296.

Guderley, H. (1994). Physiological ecology and evolution of the threespine stickleback. In *The Evolutionary Biology of the Threespine Stickleback* (ed. M. A. Bell and S. A. Foster), pp. 85-113. Oxford, UK: Oxford University Press.

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

Hagen, D. W. (1967). Isolating mechanisms in threespine sticklebacks (*Gasterosteus aculeatus*). *J. Fish. Res. B. Canada* **24**, 1637-1692.

Hansen, T. F., Carter, A. J. R. and Pelabon, C. (2006). On adaptive accuracy and precision in natural populations. *Am. Nat.* **168**, 168-181.

Hayes, J. P. and O'Connor, C. S. (1999). Natural selection on thermogenic capacity of high-altitude deer mice. *Evolution* **53**, 1280-1287.

Henderson, K. K., Wagner, H., Favret, F., Britton, S. L., Koch, L. G., Wagner, P. D. and Gonzalez, N. C. (2002). Determinants of maximal O_2 uptake in rats selectively bred for endurance running capacity. *J. Appl. Physiol.* **93**, 1265-1274.

Henriksson, P., Mandic, M. and Richards, J. G. (2008). The osmo-respiratory compromise in sculpins: Impaired gas exchange is associated with freshwater tolerance. *Physiol. Biochem. Zool.* **81**, 310-319.

Heuts, M. J. (1945). La regulation minerale en fonction de la temperature chez *Gasterosieus aculeatus*. Son importance au point de vue de la zoogeographic de l'espece. *Ann. Soc. R. Zool. Belg.* **76**, 88-99.

Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution.* Oxford, UK: Oxford University Press.

Hoffmann, E. and Borg, B. (2006). Sex differences in pectoral muscles but not in pectoral fins in the three-spined stickleback, *Gasterosteus aculeatus*. *J. Fish Biol.* **68**, 1451-1459.

Hoppeler, H. and Weibel, E. R. (1998). Limits for oxygen and substrate transport in mammals. *J. Exp. Biol.* **201**, 1051-1064.

Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biom. J.* **50**, 346-363.

Howlett, R. A., Kirkton, S. D., Gonzalez, N. C., Wagner, H. E., Britton, S. L., Koch, L. G. and Wagner, P. D. (2009). Peripheral oxygen transport and utilization in rats following continued selective breeding for endurance running capacity. *J. Appl. Physiol.* **106**, 1819-1825.

Hughes, G. M. (1984). Measurement of gill area in fishes: practices and problems. *J. Mar. Biol. Assoc. UK* **64**, 637-655.

Husak, J. F. and Fox, S. F. (2008). Sexual selection on locomotor performance. *Evol. Ecol. Res.* **10**, 213-228.
Irschick, D. J., Meyers, J. J., Husak, J. F. and Le Galliard, J. F. (2008). How does selection operate on whole-organism functional performance capacities? A review and synthesis. *Evol. Ecol. Res.* **10**, 177-196.
Jabarsyah, A., Tsuchimoto, M., Yada, O., Kozuru, Y., Miyake, T., Misima, T., Wang, Q. and Tachibana, K. (2000). Comparison of biochemical and physiological characteristics among white, pink, and red muscle fibers in carp (cultured). *Fish. Sci.* **66**, 586-593.
Jackson, D. M., Trayhurn, P. and Speakman, J. R. (2001). Associations between energetics and over-winter survival in the short-tailed field vole *Microtus agrestis*. *J. Anim. Ecol.* **70**, 633-640.
Jimenez, A. G., Dasika, S. K., Locke, B. R. and Kinsey, S. T. (2011). An evaluation of muscle maintenance costs during fiber hypertrophy in the lobster *Homarus americanus*: are larger muscle fibers cheaper to maintain? *J. Exp. Biol.* **214**, 3688-3697.
Johnston, I. A., Davison, W. and Goldspink, G. (1977). Energy metabolism of carp swimming muscles. *J. Comp. Physiol.* **114**, 203-216.
Johnston, I. A., Abercromby, M. and Andersen, O. (2005). Loss of muscle fibres in a landlocked dwarf Atlantic salmon population. *Biol. Lett.* **1**, 419-422.
Johnston, I. A., Bower, N. I. and Macqueen, D. J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. *J. Exp. Biol.* **214**, 1617-1628.
Jones, F. C., Brown, C., Pemberton, J. M. and Braithwaite, V. A. (2006). Reproductive isolation in a threespine stickleback hybrid zone. *J. Evol. Biol.* **19**, 1531-1544.
Kinsey, S. T., Locke, B. R. and Dillaman, R. M. (2011). Molecules in motion: influences of diffusion on metabolic structure and function in skeletal muscle. *J. Exp. Biol.* **214**, 263-274.
Kirkton, S. D., Howlett, R. A., Gonzalez, N. C., Giuliano, P. G., Britton, S. L., Koch, L. G., Wagner, H. E. and Wagner, P. D. (2009). Continued artificial selection for running endurance in rats is associated with improved lung function. *J. Appl. Physiol.* **106**, 1810-1818.
Konarzewski, M. and Diamond, J. (1995). Evolution of basal metabolic rate and organ masses in laboratory mice. *Evolution* **49**, 1239-1248.
Lahti, D. C., Johnson, N. A., Ajie, B. C., Otto, S. P., Hendry, A. P., Blumstein, D. T., Coss, R. G., Donohue, K. and Foster, S. A. (2009). Relaxed selection in the wild. *Trends Ecol. Evol.* **24**, 487-496.
Langerhans, R. B. and Reznick, D. N. (2009). Ecology and evolution of swimming performance in fishes: predicting evolution with biomechanics. In *Fish Locomotion: an Etho-ecological Perspective* (ed. P. Domenici and B. G. Kapoor), pp. 200-248. Enfield, NH: Science Publishers.
Losos, J. B. (2011). Convergence, adaptation, and constraint. *Evolution* **65**, 1827-1840.
Marchinko, K. B. and Schluter, D. (2007). Parallel evolution by correlated response: lateral plate reduction in threespine stickleback. *Evolution* **61**, 1084-1090.
McClelland, G. B., Dalziel, A. C., Fragoso, N. M. and Moyes, C. D. (2005). Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. *J. Exp. Biol.* **208**, 515-522.
McKinnon, J. S. and Rundle, H. D. (2002). Speciation in nature: the threespine stickleback model systems. *Trends Ecol. Evol.* **17**, 480-488.
McPhail, J. D. (1994). Speciation and the evolution of reproductive isolation in the sticklebacks (*Gasterosteus*) of south-western British Columbia. In *The Evolutionary Biology of the Threespine Stickleback* (ed. M. A. Bell and S. A. Foster), pp. 399-437. Oxford, UK: Oxford University Press.
Moyes, C. D., Mathieu-Costello, O. A., Tsuchiya, N., Filburn, C. and Hansford, R. G. (1997). Mitochondrial biogenesis during cellular differentiation. *Am. J. Physiol. Cell Physiol.* **272**, C1345-C1351.
Nespolo, R. F., Bustamante, D. M., Bacigalupe, L. D. and Bozinovic, F. (2005). Quantitative genetics of bioenergetics and growth-related traits in the wild mammal, *Phyllotis darwini*. *Evolution* **59**, 1829-1837.
Nespolo, R. F., Roff, D. A. and Fairbairn, D. J. (2008). Energetic trade-off between maintenance costs and flight capacity in the sand cricket (*Gryllus firmus*). *Funct. Ecol.* **22**, 624-631.
Nilsson, G. E. (2007). Gill remodeling in fish – a new fashion or an ancient secret? *J. Exp. Biol.* **210**, 2403-2409.
Odell, J. P., Chappell, M. A. and Dickson, K. A. (2003). Morphological and enzymatic correlates of aerobic and burst performance in different populations of Trinidadian guppies *Poecilia reticulata*. *J. Exp. Biol.* **206**, 3707-3718.
Orczewska, J. I., Hartleben, G. and O'Brien, K. M. (2010). The molecular basis of aerobic metabolic remodeling differs between oxidative muscle and liver of threespine sticklebacks in response to cold acclimation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **299**, R352-R364.
Ostlund-Nilsson, S., Mayer, I. and Huntingford, F. (2007). *Biology of the Three-Spined Stickleback*. Boca Raton, FL: CRC Press.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and the R Development Core Team (2009). nlme: linear and nonlinear mixed effects models. R package version 3.1-96. Vienna, Austria: R Foundation for Statistical Computing.
R Development Core Team (2010). R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing, http://www.R-project.org.
Rasband, W. S. (2011). ImageJ. Bethesda, MD: US National Institutes of Health, http://imagej.nih.gov/ij/.
Reichmann, H., Hoppeler, H., Mathieu-Costello, O., Vonbergen, F. and Pette, D. (1985). Biochemical and ultrastructural changes of skeletal muscle mitochondria after chronic electrical stimulation in rabbits. *Pflugers Arch.* **404**, 1-9.
Rezende, E. L., Garland, T., Chappell, M. A., Malisch, J. L. and Gomes, F. R. (2006). Maximum aerobic performance in lines of *Mus* selected for high wheel-running activity: effects of selection, oxygen availability and the mini-muscle phenotype. *J. Exp. Biol.* **209**, 115-127.
Rouleau, S., Glemet, H. and Magnan, P. (2010). Effects of morphology on swimming performance in wild and laboratory crosses of brook trout ecotypes. *Funct. Ecol.* **24**, 310-321.
Sadowska, E. T., Baliga-Klimczyk, K., Chrzascik, K. M. and Koteja, P. (2008). Laboratory model of adaptive radiation: a selection experiment in the bank vole. *Physiol. Biochem. Zool.* **81**, 627-640.
Saimoto, R. S. (1993). Reproductive and natal homing of marine threespine sticklebacks (*Gasterosteus aculeatus*). MSc thesis, University of British Columbia, Vancouver.
Sardella, B. A. and Brauner, C. J. (2007). The osmo-respiratory compromise in fish: the effects of physiological state and the environment. In *Fish Respiration and Environment* (ed. M. N. Fernandes, F. T. Rantin, M. L. Glass and B. G. Kapoor), pp. 147-165. Enfield, NH: Science Publishers.
Schaarschmidt, T. and Jurss, K. (2003). Locomotory capacity of Baltic Sea and freshwater populations of the threespine stickleback (*Gasterosteus aculeatus*). *Comp. Biochem. Physiol.* **135A**, 411-424.
Schaarschmidt, T., Meyer, E. and Jurss, K. (1999). A comparison of transport-related gill enzyme activities and tissue-specific free amino acid concentrates of Baltic Sea (brackish water) and freshwater threespine sticklebacks, *Gasterosteus aculeatus*, after salinity and temperature acclimation. *Mar. Biol.* **135**, 689-697.
Schluter, D., Marchinko, K. B., Barrett, R. D. H. and Rogers, S. M. (2010). Natural selection and the genetics of adaptation in threespine stickleback. *Philos. Trans. R. Soc. Lond. B* **365**, 2479-2486.
Scott, G. R., Egginton, S., Richards, J. G. and Milsom, W. K. (2009). Evolution of muscle phenotype for extreme high altitude flight in the bar-headed goose. *Proc. R. Soc. Lond. B* **276**, 3645-3653.
Seiler, S. M. and Keeley, E. R. (2007). Morphological and swimming stamina differences between Yellowstone cutthroat trout (*Oncorhynchus clarkii bouvieri*), rainbow trout (*Oncorhynchus mykiss*), and their hybrids. *Can. J. Fish. Aquat. Sci.* **64**, 127-135.
Suarez, R. K. (1996). Upper limits to mass-specific metabolic rates. *Annu. Rev. Physiol.* **58**, 583-605.
Taylor, C. R. and Weibel, E. R. (1981). Design of the mammalian respiratory system. 1. Problem and strategy. *Respir. Physiol.* **44**, 1-10.
Taylor, E. B. and McPhail, J. D. (1986). Prolonged and burst swimming in anadromous and freshwater threespine stickleback, *Gasterosteus aculeatus*. *Can. J. Zool.* **64**, 416-420.
Te Kronnie, G., Tatarczuch, L., Van Raamsdonk, W. and Kilarski, W. (1983). Muscle fibre types in the myotome of stickleback, *Gasterosteus aculeatus* L.; a histochemical, immunohistochmical and ultrastructural study. *J. Fish Biol.* **22**, 303-316.
Thorsen, D. H. and Westneat, M. W. (2005). Diversity of pectoral fin structure and function in fishes with labriform propulsion. *J. Morphol.* **263**, 133-150.
Tudorache, C., Blust, R. and De Boeck, G. (2007). Swimming capacity and energetics of migrating and non-migrating morphs of three-spined stickleback *Gasterosteus aculeatus* L. and their ecological implications. *J. Fish Biol.* **71**, 1448-1456.
Turner, N., Hulbert, A. J. and Else, P. L. (2006). Limits to physical performance and metabolism across species. *Curr. Opin. Clin. Nutr. Metab. Care* **9**, 691-696.
Wagner, P. D. (1996). Determinants of maximal oxygen transport and utilization. *Annu. Rev. Physiol.* **58**, 21-50.
Wagner, P. D. (2010). Limiting factors of exercise performance. *Dtsch. Z. Sportmed.* **61**, 108-111.
Wainwright, P. C., Alfaro, M. E., Bolnick, D. I. and Hulsey, C. D. (2005). Many-to-one mapping of form to function: A general principle in organismal design? *Integr. Comp. Biol.* **45**, 256-262.
Walker, J. A. (2004). Dynamics of pectoral fin rowing in a fish with an extreme rowing stroke: the threespine stickleback (*Gasterosteus aculeatus*). *J. Exp. Biol.* **207**, 1925-1939.
Walker, J. A. (2010). An integrative model of evolutionary covariance: a symposium on body shape in fishes. *Integr. Comp. Biol.* **50**, 1051-1056.
Weibel, E. R. and Hoppeler, H. (2005). Exercise-induced maximal metabolic rate scales with muscle aerobic capacity. *J. Exp. Biol.* **208**, 1635-1644.
Weibel, E. R., Taylor, C. R. and Hoppeler, H. (1991). The concept of symmorphosis: a testable hypothesis of structure function relationship. *Proc. Natl. Acad. Sci. USA* **88**, 10357-10361.
Withler, R. E. and McPhail, J. D. (1985). Genetic variability in freshwater and anadromous sticklebacks (*Gasterosteus aculeatus*) of southern British Columbia. *Can. J. Zool.* **63**, 528-533.
Zera, A. J., Sall, J. and Grudzinski, K. (1997). Flight-muscle polymorphism in the cricket *Gryllus firmus*: muscle characteristics and their influence on the evolution of flightlessness. *Physiol. Zool.* **70**, 519-529.
Zera, A. J., Potts, J. and Kobus, K. (1998). The physiology of life-history trade-offs: experimental analysis of a hormonally induced life-history trade-off in *Gryllus assimilis*. *Am. Nat.* **152**, 7-23.
Zierath, J. R. and Hawley, J. A. (2004). Skeletal muscle fiber type: influence on contractile and metabolic properties. *PLoS Biol.* **2**, e348.