PHYSIOLOGICAL ADAPTATIONS TO SWIMMING IN FISH

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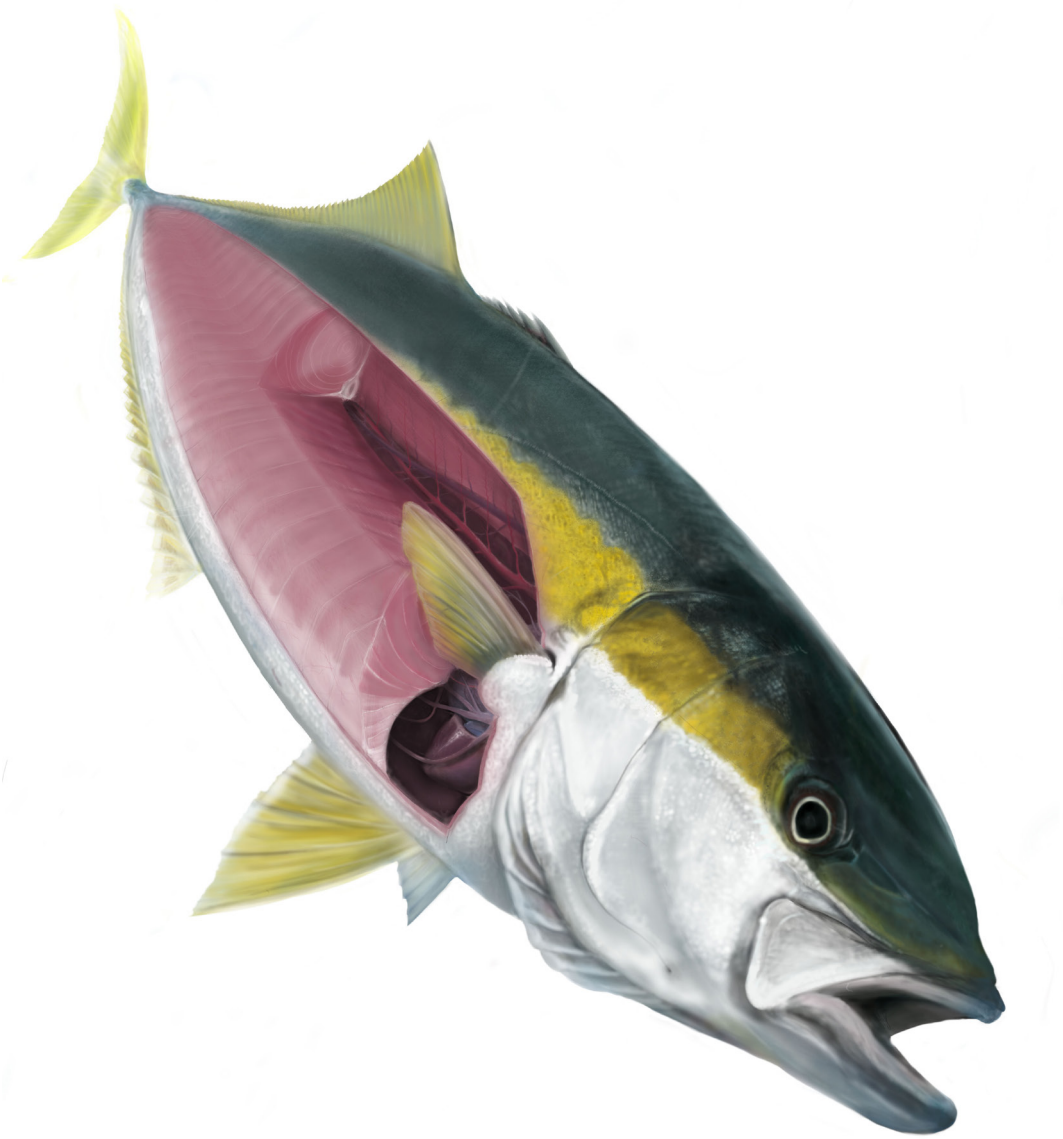

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PHYSIOLOGICAL ADAPTATIONS TO SWIMMING IN FISH

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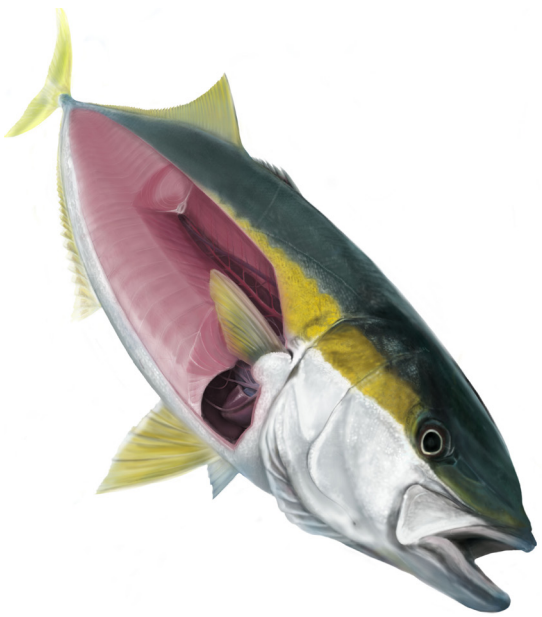

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Swimming is an integral part of the life history of many fish species as is intimately linked with their ability to express feeding and predator avoidance behaviors, habitat selection and environmental preferences, social and reproductive behaviors as well as migratory behaviors. Therefore, swimming is an important determinant factor of fitness in a true Darwinian sense and, not surprisingly, swimming performance has been often used as a measure of physiological fitness in fish. The main aim of this Research Topic is to showcase some of the current studies designed to improve our understanding of the physiological energetic and metabolic requirements of swimming and of the adaptive responses to swimming in fish.

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Editorial: Physiological Adaptations to Swimming in Fish

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Keywords: fish, performance, swimming exercise, growth, swimming economy

Editorial on the Research Topic

Physiological Adaptations to Swimming in Fish

Swimming is an integral part of the life history of many fish species as is intimately linked with their ability to express feeding and predator avoidance behaviors, habitat selection and environmental preferences, social and reproductive behaviors as well as migratory behaviors (Videler, 1993; Palstra and Planas, 2011). Therefore, swimming is an important determinant factor of fitness in a true Darwinian sense and, not surprisingly, swimming performance has been often used as a measure of physiological fitness in fish (Hammer, 2005). In the face of growing changes in the aquatic environment due to global warming and other anthropogenic influences (e.g., hydropower plants and pumping stations, pollution, destruction of essential habitats, etc.), swimming performance can become a relevant proxy for the level of fitness in our evaluation of organismal responses to environmental perturbations in wild fish populations. Changes in the locomotory capabilities of fish due to alterations in swimming performance can have important consequences at the population level in terms of individual dispersal and species abundance, reproductive success and genetic structure of the fish populations, as shown in other vertebrate groups (Hillman et al., 2014). Reduced activity levels due to swimming in captivity can also decrease their physiological fitness status or condition as it is known to occur in aquaculture, when fish cannot display their normal swimming behavior due to confinement under high densities or to insufficient water flows to induce swimming, leading to decreased fitness (both physical and reproductive), growth, survival and muscle quality, depending on the swimming characteristics of the species (Palstra and Planas, 2013). An extensive body of literature supports the notion that swimming, through the ensuing muscle contraction and activation of the cardiovascular system, affects the physiology of the fish through adaptive mechanisms that are recently beginning to be uncovered (Palstra and Planas, 2013; Rodnick and Planas, 2016). Further research efforts in this area should inform the scientific community and the public on the ability of wild fish populations to cope with environmental change and on the benefits of induced swimming for improved aquaculture production and fish welfare.

The main aim of this Research Topic is to showcase some of the current studies designed to improve our understanding of the physiological energetic and metabolic requirements of swimming and of the adaptive responses to swimming in fish. A total of 9 articles are presented, covering topics related to swimming performance in wild and aquaculture-relevant species. The first three articles report on swimming capacity and energetics in different fish species. Tudorache et al. provide a first comparison of swimming capacity and energetic profiles between European eel (*Anguilla Anguilla)* and New Zealand short-finned eel (*A. australis)*, two species that differ in the extent of their migration to their potential spawning sites. Their results indicate that European eels have higher swimming capacity but that the two species show similar energetic

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profiles during swimming. Killen et al. investigate the trade-offs associated with predator avoidance and energy balance across a range of temperatures in the golden gray mullet (*Liza aurata*). Their results show that fish that respond first to danger and that show higher escape performance are characterized by higher aerobic scope (AS) and longer recovery rates independently of temperature, suggesting that the energetic demands of vigilance, possibly due to higher brain activity, prolong physiological recovery after anaerobic exercise. Svendsen et al. report on the metabolic costs of swimming in relation to the trade-off between aerobic and anaerobic traits in gilthead sea bream (*Sparus aurata*). Their results show that fish with high maximum sustained swimming speed (U_{sus}) also exhibit high optimum swimming speed (U_{opt}) and low minimum cost of transport (COT), suggesting that high U_{sus} and minimum COT may be optimized concurrently and that burst swimming is associated with anaerobic metabolism and a substantial metabolic cost. Moreover, four other articles describe the effects of swimming on growth and performance. Skov et al. report on the effects of swimming under dietary restriction on growth in rainbow trout (*Oncorhynchus mykiss*). Their results show that trout swimming under sustained conditions and dietary restriction experience increased relative metabolic expenditure resulting in decreased specific growth rate and feed conversion ratio but without an increase in the use of protein as fuel. In contrast, Palstra et al. report that yellowtail kingfish (*Seriola lalandi*) swimming at their established U_{opt} show increased growth performance, feeding efficiency and cardiac output, in a clear example of a species benefiting from the physiological effects of swimming. Anttila et al. report on the relationship between swimming performance and cardiorespiratory performance and morphology in the context of thermal tolerance in Atlantic salmon (*Salmo salar*). Their results show that good swimmers had improved cardiorespiratory features including thicker cardiac compact layer and taller gill secondary lamella but similar thermal tolerance and that these benefits of swimming persisted after 8 months coupled with an increase in growth rate. In the last article on growth, Khan et al. report on the swimming conditions that promote optimal growth rates and the possible influence of AS in juvenile hapuku (*Polyprion oxygeneios*). Their results indicate that juvenile hapuku show a limited growth response to swimming and that AS does not appear to limit exercise-induced growth in this species. In an attempt at describing the mechanisms responsible for the physiological benefits of swimming on skeletal muscle, the paper by Morash et al. provides information on the metabolic adaptive responses to sustained swimming in rainbow trout. Their results show that sustained swimming elicits distinct changes in red and white skeletal muscle in terms of activity and expression of metabolic markers, suggestive of phenotypic plasticity in skeletal muscle. Finally, Pelster reports on the physiological function of the swimbladder of the European eel and its role in swimming during migration. In this article, the physiological role of the swimbladder, its energetic requirements and nematode infections are discussed in the light of the European eel's reproductive migration requirements. We hope that this Research Topic will be of interest to researchers in and outside the field and will stimulate them to consider including a swimming physiological perspective in their studies.

AUTHOR CONTRIBUTIONS

JP, AP, and LM all contributed equally to write this editorial.

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ORIGINAL RESEARCH ARTICLE
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Muscle metabolic remodeling in response to endurance exercise in salmonids

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Phenotypic plasticity of skeletal muscle is relevant to swimming performance and metabolism in fishes, especially those that undergo extreme locomotory feats, such as seasonal migration. However, the influence of endurance exercise and the molecular mechanisms coordinating this remodeling are not well understood. The present study examines muscle metabolic remodeling associated with endurance exercise in fed rainbow trout as compared to migrating salmon. Trout were swum for 4 weeks at 1.5 BL/s, a speed similar to that of migrating salmon and red and white muscles were sampled after each week. We quantified changes in key enzymes in aerobic and carbohydrate metabolism [citrate synthase (CS), β-hydroxyacyl-CoA dehydrogenase (HOAD), hexokinase (HK)] and changes in mRNA expression of major regulators of metabolic phenotype (AMPK, PPARs) and lipid (carnitine palmitoyltransferase, CPT I), protein (aspartate aminotransferase, AST) and carbohydrate (HK) oxidation pathways. After 1 week of swimming substantial increases were seen in AMPK and PPARα mRNA expression and of their downstream target genes, CPTI and HK in red muscle. However, significant changes in CS and HK activity occurred only after 4 weeks. In contrast, there were few changes in mRNA expression and enzyme activities in white muscle over the 4-weeks. Red muscle results mimic those found in migrating salmon suggesting a strong influence of exercise on red muscle phenotype. In white muscle, only changes in AMPK and PPAR expression were similar to that seen with migrating salmon. However, in contrast to exercise alone, in natural migration HK decreased while AST increased suggesting that white muscle plays a role in supplying fuel and intermediates possibly through tissue breakdown during prolonged fasting. Dissecting individual and potentially synergistic effects of multiple stressors will enable us to determine major drivers of the metabolic phenotype and their impacts on whole animal performance.

Keywords: endurance exercise, salmonids, muscle remodeling, metabolism, migration, fuel selection

INTRODUCTION

Plasticity of skeletal muscle is integral to the physiological response of fishes to exercise. With chronic contraction there are a host of transcriptional changes leading to phenotypic plasticity of the muscle fibers that help ensure adequate oxygen and substrate delivery for ATP production to fuel the dynamic metabolism of this tissue (For review see McClelland, 2004). How muscle metabolism responds to exercise depends upon recruitment of different fiber-types (red vs. white), which is a function of the type and duration of the exercise (sprint vs. endurance; acute vs. chronic). Sub-maximal or low-intensity chronic exercise stimulates an increase in aerobic capacity (Johnston and Moon, 1980; Farrell et al., 1990) most likely through a number of molecular events involving a series of nuclear transcription factors such as nuclear respiratory factor (NRF) 1 and 2 and peroxisome proliferators-activated receptors (PPAR)-α, −β/δ, −γ and PPAR-γ cofactor (PGC)-1α that are implicated in mitochondria biogenesis and metabolic remodeling in mammalian muscle (Price et al., 2000; Baar et al., 2002). Data supporting a role for some of these factors in muscle remodeling from swim trained fishes are few and still equivocal (McClelland et al., 2006; LeMoine et al., 2010).

Submaximal chronic swimming is predicted to induce expression of key transcription factors such as PPARs. PPARs are nuclear receptors that can be activated by either elevated cellular levels of monounsaturated or polyunsaturated fatty acid ligands that, when bound, induce the PPARα-mediated expression of genes involved in the metabolism of lipids (Price et al., 2000).

Another main regulator of muscle energetic homeostasis is AMP-activated protein kinase (AMPK). AMPK works concurrently to stimulate fatty acid oxidation and glucose uptake in liver and muscle while inhibiting lipogenesis (Winder and Hardie, 1999). As a regulator of cellular energy production AMPK interacts with many of the regulatory centers of the major metabolic pathways. For example, AMPK stimulation increases PPARα and PGC-1α expression, citrate synthase activity (TCA cycle), hexokinase expression (glycolysis), carnitine palmitoyltransferase (CPT) I and β-hydroxyacl-CoA dehydrogenase (HOAD) (fatty acid

oxidation) and cytochrome *c* (electron transport chain). Taken together, AMPK acts to stimulate ATP generating processes, particularly during exercise and fasting when an up-regulation of fat and carbohydrate oxidation is warranted.

Protein oxidation plays a relatively small role in ATP production until faced with extreme conditions. In migrating salmon, for example, protein oxidation (from muscle breakdown) begins to account for a growing proportion of the fish's metabolic fuel as lipid stores are depleted (Duncan and Tarr, 1958; Idler and Bitners, 1958; French et al., 1983). Aspartate transaminase (AST), which produces oxaloacetate for the TCA cycle (Hochachka and Somero, 1984), can be used as an indicator of protein oxidation capacity. Protein oxidation in satiated acutely exercised fish does not increase with swimming duration and contributes little to energy production (Alsop and Wood, 1997).

Muscle phenotype varies naturally in many fish species due to many factors including exercise. Probably the most extreme example of endurance exercise is that of the seasonal migration in Pacific salmon (10–2000 km), which requires specific metabolic alterations to ensure arrival at spawning grounds. However, the stressors encountered with spawning migrations are multifactorial including salinity changes, sexual maturation, and the cessation of feeding along side of chronic exercise. Current work on muscle metabolism in these animals indicates that there are specific temporal changes in transcription factors and key regulators of lipid, protein and carbohydrate oxidation (Morash et al., 2013), but it is unclear how exercise interacts with other influences such as fasting to influence this pattern. Recently we have uncovered some responses of muscle to long-term fasting in the closely related rainbow trout (Morash and McClelland, 2011). In the current study, we investigate long term exercise in fed rainbow trout to determine its effect(s) on the phenotypic plasticity red and white muscle.

Using rainbow trout (*Oncorhynchus mykiss*) as our model we examined the metabolic changes that take place during long-term exercise in fed fish. Specifically this research will determine spatial and temporal changes in transcription and activities of key metabolic enzymes central to energy metabolism in rainbow trout. We will relate these changes to those seen in trout with fasting Morash and McClelland (2011) with the goal of understanding their relative roles to changes seen in muscle with migration in a related salmonid, the Pacific salmon (Morash et al., 2013).

MATERIALS AND METHODS

EXPERIMENTAL SPECIES AND EXERCISE REGIME

All procedures were approved by the McMaster University Animal Research Ethics Board. Rainbow trout were obtained from a local trout hatchery (Humber Springs, Orangeville, ON), kept in 500 L tanks with circulating dechloriniated Hamilton tap water at 12°C and fed a commercial diet (Profishent Classic Floating Trout Grower, Martin Mills, Elmira, ON) twice daily to satiation. The exercised groups were introduced to the swim tunnel 1 week to acclimate prior to the beginning of exercise while control fish were maintained under routine conditions for at least 1 week prior to sacrifice. Fish were then exercised for 1, 2, or 4 weeks at 1.5 BL s^{-1} (approximately 50% U_{crit}) for 23.5 h per day. Water flow was stopped twice for 15 min when the exercised trout were fed to satiation the same commercial trout feed as controls. Control fish were sampled after 1 week in routine conditions while swum fish were sampled after 1, 2, and 4 weeks of swimming. Both groups were euthanized by a blow to the head and then severing of the spinal cord. Length and weight were recorded. Condition factor (CF) was calculated using the following equation,

$$CF = 100w/l^3 \quad (1)$$

where w is the weight of the fish in grams and l is the length of the fish in centimeters. Red and white muscle samples were excised and immediately frozen in liquid nitrogen and stored at −80°C until analysis.

RNA EXTRACTION AND cDNA SYNTHESIS

Frozen tissues were powdered in a liquid N_2-chilled mortar and pestle. Total RNA was extracted from each tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA) based on the acid guanidinium thiocyanate-phenol-chloroform extraction method. RNA was quantified by UV spectroscopy at 260 nm and then diluted to 0.5 μg/μl. cDNA was synthesized using 1 μg of DNase (Invitrogen, Carlsbad, CA) treated mRNA with SuperScript RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA) as described previously (Morash et al., 2008).

POLYMERASE CHAIN REACTION (PCR) AND SEQUENCING

For each gene, available sequences from other fish species and mammals were aligned and PCR primers were designed using Primer3 software (Rozen and Skaletsky, 2000) within highly conserved regions. Each gene segment was amplified by PCR using 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.5 μM of each forward and reverse primer (**Table 1**), 1 unit of Taq polymerase (Fermentas, Burlington, Onatrio, Canada) and 1× Taq amplification buffer. All PCR products were verified through separation by gel electrophoresis using DNA size standards. Products were then purified using QiaQuick gel extraction kit (Qiagen, Mississauga, Ontario, Canada) and directly sequenced at the Mobix Lab (McMaster University) to ensure the proper sequence was being amplified.

Table 1 | Primer sequences used for real time PCR analysis.

Gene	5′ to 3′ Forward primer	5′ to 3′ Reverse primer	T_m °C	Size (bp)
PPARα	ccaagttcagtttgccatga	attggggaagaggaaggtgt	60	173
PPARβ	ctggagctggatgacagtga	gtcagccatcttgttgagca	60	195
CPT Iβ1	gatgttccgtgagggtagga	ttgtcttgcatggctctgac	58	80
CPT Iβ2	gccgcaaactagagagagga	cccgtagtacagccacacct	58	199
CPT Iα1a	atgaggaatgccctcaagtg	gcttcctgccagagaacaac	58	120
CPT Iα1b	cgcttcaagaatggggtgat	caaccacctgctgtttctca	58	187
CPT Iα2	ccgttcctaacagaggtgct	acactccgtagccatcgtct	58	154
HK	ctgggacgctgaagaccaga	cggtgctgcatacctccttg	58	159
AST	gacctgtggctttgacttcc	gcaatctccttccactgctc	58	135
AMPK	actgtgttccgttggacagg	tcaatcatgagggcatcaaa	58	272
EF1α	cattgacaagagaaccattga	ccttcagcttgtccagcac	58	94

mRNA QUANTIFICATION BY REAL-TIME PCR

The expression of each mRNA was quantified using real time PCR with SYBR green plus ROX as a reference dye on a Stratagene Mx3000P (Stratagene, Texas, USA) real-time PCR system. Each 25 μl reaction contained 12.5 μL SYBR green mix, 1 μL each of forward and reverse primers (5μM), 5.5 μL of DNase/RNase free water and 5 μL of 5× diluted cDNA (a no template control was included to ensure there was no contamination). Real-time PCR primers were designed using Primer3 software (Rozen and Skaletsky, 2000; **Table 1**). The thermal program included 3 min at 95°C, 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 30 s. A dissociation curve was performed to ensure only one PCR product was being amplified. Standard curves were constructed for each gene using serial dilutions of stock cDNA to account for any differences in amplification efficiencies. All samples were normalized to the housekeeping gene, EF1-α whose expression did not change significantly between time points.

ENZYME ANALYSIS

All assays were performed in triplicate at room temperature in 96-well format using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA), and data was collected using Softmax Pro 4.7.1 software (Molecular Devices, Sunnyvale, CA). Frozen tissues were powdered using a liquid N_2 chilled mortar and pestle and homogenized in 20 volumes of ice-cold homogenization buffer (100 mM potassium phosphate, 5 mM EDTA and 0.1% Triton at pH 7.2) using a glass on glass homogenizer chilled on ice. Homogenized samples were kept on ice prior to enzymatic analysis.

Citrate Synthase (CS)

CS was measured according to previously published protocols (McClelland et al., 2005). Briefly, the CS assay buffer contained (in mM) 20 TRIS (pH 8.0), 0.1 DTNB and 0.3 acetyl-CoA. The reaction was initiated by the addition of 0.5 mM oxaloacetate and absorbance was measured for 5 min at 412 nm. Control samples were assayed without oxaloacetate to control for background hydrolase activity.

β-hydroxyacyl-CoA Dehydrogenase (HOAD)

HOAD was measured according to previously published methods (McClelland et al., 2005) and consisted of (in mM) 50 imidazole (pH7.4), 0.1 acetoacetyl-CoA, 0.15 NADH and 0.1% Triton X-100 at 340 nm.

Hexokinase (HK)

The HK assay was modified from Houle-Leroy et al. (2000) for use in fish. The assay buffer contained (in mM) 4 ATP, 10 $MgCl_2$, 0.5 NADP, 1 U glucose-6-phosphate dehydrogenase, in 50 HEPES (pH 7.0). The reaction was initiated by the addition of 5 mM D-glucose (omitted in control reactions).

STATISTICAL ANALYSIS

All statistical analyses were performed using SigmaStat v3.5 and Sigmaplot v12.5 (Systat Software Inc., San Jose, CA). All data were tested for normality and equal variance prior to performing One-Way ANOVA and Holm-Sidak post tests to test for significance between tissues and treatments. Significance level was set at $p < 0.05$.

RESULTS

CONDITION OF TROUT DURING EXPERIMENTAL TRIALS

Control trout showed no significant change in length, body weight or condition factor (CF) over the course of 4 week experimental period (**Table 2**). The exercised groups all showed a significant increase in their CF after their respective exercise regime. After 1 week of training, there was no significant change in length or weight of the fish, however, in combination the small changes lead to a significant increase in CF from 1.06 to 1.27. Two weeks of exercise significantly increased the mass of the fish but not the length leading to a CF o 1.25. By 4 weeks, exercise led to increases in both length and weight by 10 and 63%, respectively (**Table 2**; $p < 0.05$).

GENE EXPRESSION

After 1 week of chronic exercise, trout showed large and significant induction in mRNA expression for AMPK (10-fold), PPARα (36-fold), HK (15-fold), AST (5-fold) and the CPT 1 isoforms β1a, β1b, α1a, α1b (13–38-fold) in red muscle (**Figures 1A,B**; $p < 0.05$). Expression of all of these genes returned to control levels by week 2 of training except AST remained elevated and CPT Iα2 where expression increased only at this time point (**Figures 1A,B**; $p < 0.05$). Interestingly, the transcription factor PPARβ showed no significant change in mRNA expression with exercise (**Figure 1A**).

The pattern of gene expression was distinct in white muscle with PPARα mRNA expression doubling but only after 4 weeks of exercise (**Figure 2A**; $p < 0.05$). In contrast, PPARβ mRNA expression was 50% lower after 1, 2, and 4 weeks of exercise

Table 2 | Physical characteristics of control trout and after 1, 2, or 4 weeks of exercise.

	Initial	Final
CONTROL		
Length (cm)	16.10 ± 0.20	16.17 ± 0.39
Weight (g)	43.95 ± 2.09	47.75 ± 4.54
CF	1.05 ± 0.02	1.11 ± 0.05
1 WEEK EXERCISE		
Length (cm)	13.79 ± 0.39	14.12 ± 0.67
Weight (g)	28.54 ± 0.79	30.68 ± 5.15
CF	1.06 ± 0.06	1.27 ± 0.08*
2 WEEKS EXERCISE		
Length (cm)	15.16 ± 0.08	15.50 ± 0.39
Weight (g)	34.20 ± 1.03	45.13 ± 3.24*
CF	1.03 ± 0.04	1.21 ± 0.04*
4 WEEKS EXERCISE		
Length (cm)	16.50 ± 0.35	18.12 ± 0.38*
Weight (g)	46.17 ± 2.64	75.15 ± 5.08*
CF	1.02 ± 0.03	1.25 ± 0.04*

Values are means ± SE. n = 5. Asterisks indicate significance between initial and final values. CF, condition factor.

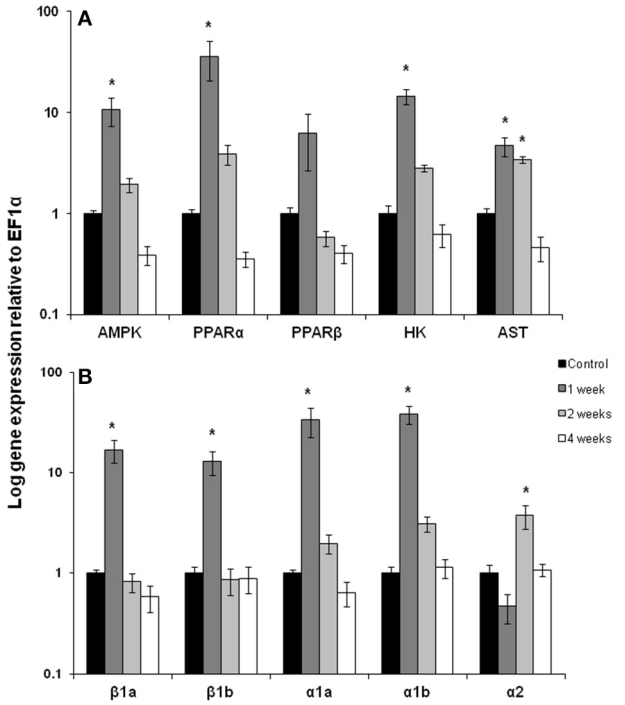

FIGURE 1 | Red muscle mRNA expression of (A) AMPK, AMP kinase; PPARα and β, peroxisome proliferator-activated receptor; HK, hexokinase; AST, aspartate amino transferase. (B) carnitine palmitoyl-carnitine (CPT)I β1a, β1b, α1a, α1b, α2 in control, 1, 2, and 4 week exercised rainbow trout. Values are means ± *SE*. $n = 4$. Asterisk indicates significance between time points for each individual isoform.

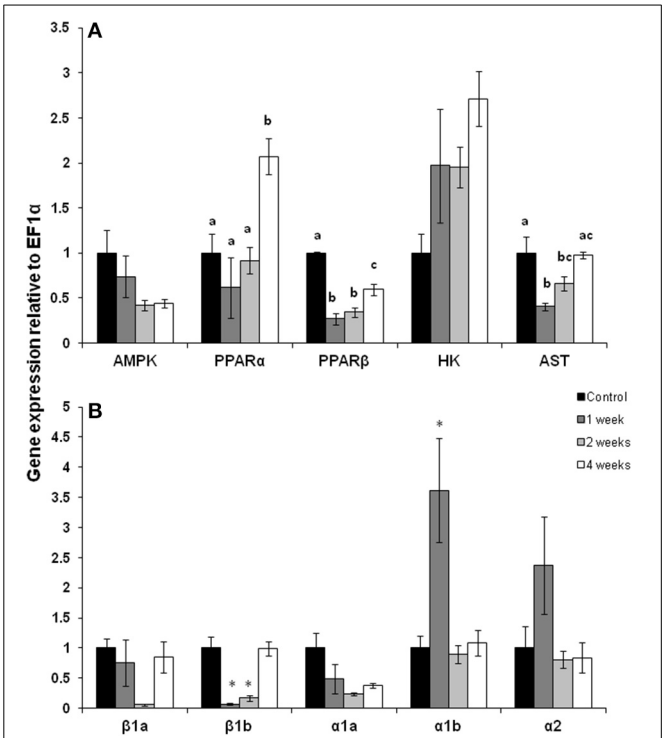

FIGURE 2 | White muscle mRNA expression of (A) metabolic pathway regulators and (B) CPT I isoforms in (A) AMPK, AMP kinase; PPARα and β, peroxisome proliferator-activated receptor; HK, hexokinase; AST, aspartate amino transferase. (B) Carnitine palmitoyl-carnitine (CPT)I β1a, β1b, α1a, α1b, α2 in control, 1, 2, and 4 week exercised rainbow trout. Values are means ± *SE*. $n = 4$. Asterisks or letters indicate significance between time points for each individual isoform.

(**Figure 2A**; $p < 0.05$). There was a trend for AMPK expression to decrease throughout the training period but this decline was not statistically significant. AST mRNA expression fell by 50% after 1 week of exercise, remained low at 75% of control expression after 2 weeks but returned to control expression levels by 4 weeks of training (**Figure 2A**; $p < 0.05$). The expression of CPT I isoforms was variable with CPT Iβ1b decreasing at weeks 1 and 2, while CPT Iα1b increased by 350% at week 1 **Figure 2B**; $p < 0.05$).

ENZYME ACTIVITY

The activity of HOAD in both red and white muscle was unaffected by chronic swimming (**Figure 3A**). CS activity did not change in red muscle until 4 weeks of training when it increased significantly from controls (**Figure 3B**; $p < 0.05$). In white muscle, CS was significantly lower after 1 and 2 weeks of exercise (**Figure 3B**; $p < 0.05$). HK activity significantly increased in both red and white muscle after 4 weeks of exercise when compared to 1 and 2 week exercised fish (**Figure 3C**; $p < 0.05$).

DISCUSSION

Chronic swimming in fed trout leads to a metabolic remodeling of skeletal muscle that varied both spatially between fiber-types and temporally over a 4 week period. Red muscle showed a very large (up to 10×) transcriptional response after only a week of exercise, but changes in maximal activities of enzymes occurred only after 4 weeks of exercise. In contrast, white muscle gene expression was generally reduced throughout the duration of training with the exception of an increase in PPARα at 4 weeks. Similar to transcription, there were either no changes or decreased enzymatic activity in white muscle, except for HK which increased only after 4 weeks. Exercise is an important stimulator of grown in fish and trout did show increased growth by 4 weeks of training. The duration and intensity of the exercise in this study closely mimics that of migrating salmon. However, the extent to which individual factors of migration, such as endurance exercise and fasting, affect muscle physiology and metabolism is currently unknown. Although exercise leads to muscle remodeling in trout, changes that occur with migration likely result from a combination of multiple stressors including muscle contraction and fasting.

During the first week of chronic swimming, red muscle mRNA expression was significantly elevated for many of the genes measured (**Figure 1**). In particular, two of the main regulators of lipid oxidation, AMPK and PPARα, (**Figure 1A**) increased, along with their downstream targets. PPARα is likely responsible (Price et al., 2000) for the increase in CPT I mRNA expression at the same time point, while AMPK plays a role in stimulating gene expression from both fatty acid (CPT I) and glycolytic pathways (HK) (**Figures 1A,B**). These modifications suggest that the red muscle is predominantly geared toward lipid oxidation to fuel muscle contraction, a common response to exercise training (Johnston

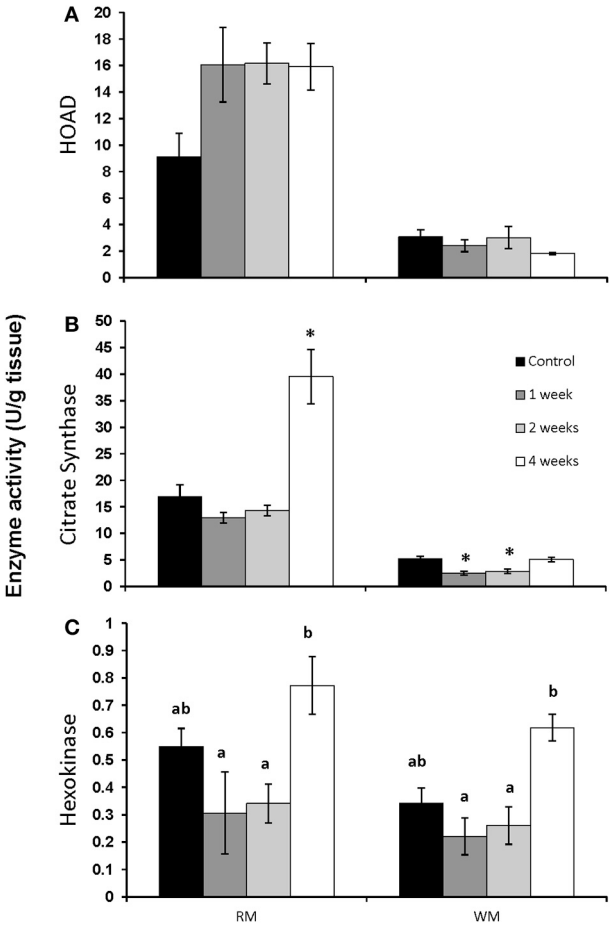

FIGURE 3 | Maximal enzyme activities (apparent Vmax) in red and white muscle from control, 1, 2, and 4 week exercised rainbow trout. **(A)** β-hydroxyacyl-CoA dehydrogenase (HOAD), **(B)** citrate synthase and **(C)** hexokinase. Values are means ± *SE*. $n = 4$. Asterisks represent significantly different from controls, and bars that do not share a letter are significantly different from each other within each tissue.

and Moon, 1980; Farrell et al., 1990). At the same time, changes in white muscle gene expression were more variable, showing no significant changes due to swim training, except for CPT I α1b. Similarly, the expression and activity of important fatty acid oxidation genes and proteins were also highest in red muscle in migrating salmon during the first week of migration while they were still in the ocean traveling to the river mouth (Morash et al., 2013). Indeed this may be a common transcriptional response to muscle contraction in fishes. Zebrafish also show a stimulation of muscle transcription during the early stages of swim training (LeMoine et al., 2010). In contrast, long-term fasting in trout led to little change in the expression of PPARα or PPARβ in red and white muscle (Morash and McClelland, 2011). However, it is unclear if transcription is stimulated immediately upon cessation of feeding or how combined fasting and exercise interact at the level of skeletal muscle.

The up-regulation of metabolic regulators, AMPK and PPARα, corresponded to induction in the expression of their downstream targets and suggests that changes in their transcription with exercise, but the role of post-translational modifications of these regulators in unclear. The expression and downstream effects of AMPK and PPARs in fish are still not well characterized. However, recent research has found that increased AMPK activity in exercising trout muscle facilitates downstream target gene expression (Magnoni et al., 2014). Furthermore, whole genome duplication events have resulted in multiple isoforms of factors such as PPARs (and likely AMPK), many of which have yet to be characterized (Batista-Pinto et al., 2005; Leaver et al., 2005, 2007). Large scale transcriptomic analysis of muscles from resting and exercising fish, including migrating salmon, are emerging in the literature and will help to clarify our understanding of these molecular mechanisms (Miller et al., 2009; Palstra et al., 2013). However, salmon appear to express the same multiple paralogs of CPT I originally characterized in trout (Morash et al., 2010, 2013). CPT I is transcriptionally regulated by PPARα in mammals (Price et al., 2000), but appears to be more complex in fish as the expression of CPT I α1b isoform in white muscle was activated independently of PPARα expression. CPT I may respond independently to stresses such as exercise, migration or fasting.

The significant increase in AMPK, PPARα and the CPT I isoforms seen here in trout, suggests that the capacity for fatty acid oxidation would be increased in the red muscle of these well fed, exercising fish. The increase in an enzyme used as a marker of β-oxidation capacity (HOAD) showed an almost doubling in activity within the first week of exercise, although this increase did not reach statistical significance (**Figure 3A**). The effects of short-term chronic exercise may be influencing the mRNA expression and enzyme activity during the early stages of migration (Morash et al., 2013). During the initial phase of migration, salmon have large lipid stores and therefore the available lipid supply should be similar to fed trout (French et al., 1983). Moreover, the low intensity swimming during our exercise protocol and early in migration would be primarily powered by lipid and protein oxidation (Johnston and Moon, 1980; French et al., 1983; Lauff and Wood, 1996). The increased expression of AST mRNA expression in the first week of chronic exercise in red muscle (**Figure 1A**) may serve to support this protein oxidation. In contrast there was no increase in AST mRNA expression in white muscle likely due to a low contribution to low intensity chronic swimming (Rome et al., 1984). Similar changes in AST mRNA expression in both exercised trout and migrating salmon red muscle suggest that protein oxidation in these tissues may be most affected by exercise and not fasting (Morash et al., 2013). In contrast, AST mRNA expression was 10 times higher in the white muscle of migrating salmon during the first week, which is potentially a result of fasting during the migration or a reliance on periodic burst swimming.

The initial induction of transcription seen early on in swim training were largely absent by 2 and 4 weeks and in some cases even reduced below constitutive expression levels in white muscle of trout. Similar to other fishes (LeMoine et al., 2010) and mammals (Mathai et al., 2008; Perry et al., 2010) changes in transcription and protein expression are temporally separated. By 4 weeks of swim training CS activity more than doubled in red muscle (**Figure 3**) consistent with past studies on fishes showing that mitochondrial biogenesis can occur between 1 and 4 weeks of training (Farrell et al., 1990; McClelland et al., 2006;

Palstra et al., 2010). Similarly, transcript levels were highest at early stages of migration with the exception of red muscle AMPK mRNA expression which increases later in migration, as does HK expression and activity (Morash et al., 2013). Furthermore, there is an increase in AST expression in white muscle of migrating salmon as they begin to catabolize and degrade muscle proteins after long-term swimming in the fasted state (Mommsen et al., 1980; French et al., 1983).

We have discussed the effects of chronic exercise on the phenotypic plasticity of skeletal muscle in rainbow trout and compared these to wild migrating Pacific salmon in an attempt to elucidate the individual effects of exercise on the metabolic alterations in the muscle that occur in these populations (Morash et al., 2013). While there are several similarities and differences in responses we are limited in our conclusions as migration is a multifactorial process that is influenced by changes in salinity, sexual maturation, fasting, and exercise. However, our data sheds some light on the individual effect of long term exercise in salmonids that may be contributing to the large scale metabolic changes in migrating salmon.

In conclusion, low intensity chronic exercise results in phenotypic plasticity of skeletal muscle, predominantly in red muscle with little effect on mRNA expression or enzyme activity of target enzymes in white muscle. While we found mRNA expression of transcription factors and their downstream targets to be induced during the first week of exercise while enzyme activity changes occurred later on to primarily increase muscle capacity for lipid oxidation and aerobic metabolism. Changes in markers of protein oxidation suggest that proteins may contribute to intermediate supply for the TCA cycle to fuel oxidative metabolism in the red muscle. There was a similar temporal transcriptional response in the red muscle of migrating salmon indicating that exercise may dominate the regulation of metabolism during early stages of migration. After 4 weeks of exercise there was an increase in mitochondrial biogenesis in the red muscle of trout as a result of exercise training. We did not find any changes in white muscle after 4 weeks of exercise unlike the white muscle during the final stages of migration in salmon where there is an increase in glycolytic enzyme and protein oxidation capacity. This is likely a result of long-term fasting and the depletion of lipid stores. Understanding the role of exercise in the remodeling of skeletal muscle has important implications for unraveling the complex physiological changes that occur in naturally migrating species but also for improving aquaculture practices to maximize muscle growth and fish health.

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Intraspecific variation in aerobic and anaerobic locomotion: gilthead sea bream (*Sparus aurata*) and Trinidadian guppy (*Poecilia reticulata*) do not exhibit a trade-off between maximum sustained swimming speed and minimum cost of transport

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Intraspecific variation and trade-off in aerobic and anaerobic traits remain poorly understood in aquatic locomotion. Using gilthead sea bream (*Sparus aurata*) and Trinidadian guppy (*Poecilia reticulata*), both axial swimmers, this study tested four hypotheses: (1) gait transition from steady to unsteady (i.e., burst-assisted) swimming is associated with anaerobic metabolism evidenced as excess post exercise oxygen consumption (EPOC); (2) variation in swimming performance (critical swimming speed; U_{crit}) correlates with metabolic scope (MS) or anaerobic capacity (i.e., maximum EPOC); (3) there is a trade-off between maximum sustained swimming speed (U_{sus}) and minimum cost of transport (COT_{min}); and (4) variation in U_{sus} correlates positively with optimum swimming speed (U_{opt}; i.e., the speed that minimizes energy expenditure per unit of distance traveled). Data collection involved swimming respirometry and video analysis. Results showed that anaerobic swimming costs (i.e., EPOC) increase linearly with the number of bursts in *S. aurata*, with each burst corresponding to 0.53 mg O_2 kg^{-1}. Data are consistent with a previous study on striped surfperch (*Embiotoca lateralis*), a labriform swimmer, suggesting that the metabolic cost of burst swimming is similar across various types of locomotion. There was no correlation between U_{crit} and MS or anaerobic capacity in *S. aurata* indicating that other factors, including morphological or biomechanical traits, influenced U_{crit}. We found no evidence of a trade-off between U_{sus} and COT_{min}. In fact, data revealed significant negative correlations between U_{sus} and COT_{min}, suggesting that individuals with high U_{sus} also exhibit low COT_{min}. Finally, there were positive correlations between U_{sus} and U_{opt}. Our study demonstrates the energetic importance of anaerobic metabolism during unsteady swimming, and provides intraspecific evidence that superior maximum sustained swimming speed is associated with superior swimming economy and optimum speed.

Keywords: aerobic metabolic scope, anaerobic capacity, burst swimming, excess post exercise oxygen consumption, intraspecific variation and trade-off, locomotion, maximum sustained swimming speed, minimum cost of transport

INTRODUCTION

Variation in locomotor performance and metabolism is linked to fitness, because both traits are often coupled with important behaviors such as predator evasion, prey capture, reproduction,

Abbreviations: EPOC, Excess post exercise oxygen consumption; BL, Body length; COT, Cost of transport; COT_{min}, Minimum cost of transport; Exercise MO_2, Metabolic rate measured in swimming fish (i.e., instantaneous metabolic rate); MO_2, Metabolic rate; $MO_{2active}$, Active metabolic rate defined as the maximum metabolic rate maintained for 0.5 h; MO_{2max}, Maximum metabolic rate defined as the maximum metabolic rate measured at increasing swimming speeds; $MO_{2routine}$, Routine metabolic rate defined as the average metabolic rate in fish swimming at 0.5 BL s^{-1}; MO_{2stand}, Standard metabolic rate (i.e., *a* in Equation 2); MO_{2sus}, Maximum sustained metabolic rate defined as the maximum metabolic rate (over 0.5 h) without any EPOC (i.e., no influence of anaerobic metabolism); Total MO_2, Exercise MO_2 and EPOC combined as an estimate of the total metabolic swimming cost; U_{active}, Swimming speed associated with the active metabolic rate ($MO_{2active}$); U_{crit}, Critical swimming speed; U_{max}, Swimming speed associated with the maximum metabolic rate (MO_{2max}); U_{opt}, Optimum swimming speed defined as the speed that minimizes energy expenditure per unit of distance traveled; U_{sus}, Maximum sustained swimming speed defined as the maximum recorded swimming speed (over 0.5 h) without any EPOC (i.e., no influence of anaerobic metabolism).

migration, and dominance (Clobert et al., 2000; Walker et al., 2005; Langerhans, 2009a; Leis et al., 2009; Eliason et al., 2011; Seebacher et al., 2013; Wilson et al., 2013; Burnett et al., 2014; Killen et al., 2014; Scantlebury et al., 2014). Intraspecific variation in locomotor performance and metabolism is repeatable across time and environments (Chappell and Odell, 2004; Claireaux et al., 2005, 2007; Oufiero and Garland, 2009; Norin and Malte, 2011, 2012; Careau et al., 2014) and may be heritable and/or trans-generational (Rønning et al., 2007; Dalziel et al., 2011, 2012; Dalziel and Schulte, 2012; Gore and Burggren, 2012; McKenzie et al., 2013; Mattila and Hanski, 2014), indicating that traits related to locomotor performance and metabolism are subjected to natural selection and could evolve over time.

Performance trade-offs are central to understanding the vast phenotypic variation found among species, populations, and individuals. Trade-offs may occur when two antagonistic traits cannot be optimized simultaneously, because the two traits pose conflicting demands on the same design feature (Damme et al., 2002). Consequently, excellence in one trait will come at the cost of performance in the other trait (Vanhooydonck et al., 2014). Hence, an organism may specialize in one trait at the cost of the other, in which case a trade-off may cause phenotypic differentiation (DeWitt and Scheiner, 2004; Konuma and Chiba, 2007; Herrel et al., 2009). Alternatively, the conflicting demands may result in organisms performing sub-optimally for both traits and therefore, constrain evolution (Lewontin, 1978; Arnold, 1992). In fish, there is evidence of a trade-off between endurance capacity and sprint speed (Langerhans, 2009b; Oufiero et al., 2011); however, the trade-off is not ubiquitous at the whole-organism level (Wilson et al., 2002; Vanhooydonck et al., 2014; Fu et al., 2015).

Levels of swimming exercise in fishes have been divided into three categories on the basis of the time a given speed can be maintained before the onset of fatigue (Beamish, 1978): sustained (more than 200 min), prolonged (20 s to 200 min) and burst swimming (less than 20 s). In many teleosts, the segmented myotomal musculature is distinctively divided into red oxidative (slow-twitch) muscles and white glycolytic (fast-twitch) muscles. Red muscles are powered by oxidative phosphorylation, whereas white muscles are largely powered by anaerobic utilization of phosphocreatine, ATP and glycogen. At sustainable swimming speeds, the red musculature is powering propulsion, whereas white musculature is increasingly recruited for propulsion at faster speeds. Employing white musculature for burst-assisted swimming typically involves significant physiological perturbations including decreasing levels of intracellular pH (Lurman et al., 2007) and muscle glycogen (Peake and Farrell, 2004), and increasing levels of lactate (Martínez et al., 2004; Peake and Farrell, 2004) and inorganic phosphate (Lurman et al., 2007) indicating a non-steady state and partial reliance on anaerobic metabolism. While metabolic locomotor cost during sustained swimming has received considerable attention (Brett, 1964; Steinhausen et al., 2005; Ohlberger et al., 2006; Svendsen et al., 2013), the metabolic cost during non-steady burst swimming remains poorly understood. Metabolic locomotor cost during sustained swimming can be estimated using measurements of instantaneous oxygen consumption rates (exercise MO_2), whereas metabolic cost during unsustainable swimming can be estimated by combining exercise MO_2 with excess post exercise oxygen consumption (EPOC). The presence of EPOC is considered evidence of anaerobic activity in intact fish (Beamish, 1978), with many of the physiological perturbations related to anaerobic metabolism cleared during the period associated with EPOC (Peake and Farrell, 2004). In striped surf perch (*Embiotoca lateralis*), a labriform swimmer, there is a linear relationship between the number of bursts and EPOC, with EPOC constituting 25% of the total swimming costs (total MO_2; i.e., exercise MO_2 and EPOC combined) on average (Svendsen et al., 2010). In contrast, the metabolic cost of burst swimming in axial swimmers is largely unknown (Puckett and Dill, 1984; Farrell, 2007).

Standard metabolic rate (MO_{2stand}) is a basic maintenance requirement measured as the minimum rate of oxygen consumption of postprandial unstressed animals at rest, below which physiological function is impaired. Long-term energy demands for swimming, food acquisition and treatment, regulation owing to environmental perturbations, and reproduction are additional to standard metabolism. These demands are met within the range set by the maximum metabolic rate (MO_{2max}) (Priede, 1985). The difference between MO_{2stand} and MO_{2max} is termed the metabolic scope (MS). Because MS is strongly influenced by environmental variables, including temperature and oxygen availability, MS is predicted to be a major physiological variable in relation to climate change and aquatic hypoxia (Claireaux and Lefrançois, 2007; Chabot and Claireaux, 2008; Guderley and Pörtner, 2010; Pörtner, 2010; Pörtner and Peck, 2010; Di Santo, 2015). Nevertheless, intraspecific relationships between MS and other important physiological traits have rarely been explored in detail. MS and swimming performance correlate positively in intraspecific comparisons involving disparate populations of Atlantic silverside (*Menidia menidia*) (Arnott et al., 2006) and rainbow trout (*Oncorhynchus mykiss*) (Claireaux et al., 2005), but it remains uncertain to what extent the relationship is found in other species.

Locomotor performance and associated metabolic costs are often coupled with life history traits, which may involve trade-offs related to growth and MO_{2stand} (Arnott et al., 2006; Rouleau et al., 2010). Recently, it was suggested that a trade-off between maximum sustained swimming speed (U_{sus}) and minimum cost of transport (COT_{min}) may be driving morphological diversity in axial swimmers including teleosts and cetaceans (Tokić and Yue, 2012). The trade-off assumes constraints in optimizing both U_{sus} and COT_{min} and suggests that aquatic species optimize either U_{sus} or COT_{min}. Tokić and Yue (2012) applied the trade-off to models of morphological variation and reported congruent morphological variation in a number of extant aquatic species. While the trade-off may explain interspecific morphological variation, the trade-off has not been examined empirically at the intraspecific level. Likewise, it is not known if intraspecific diversity in U_{sus} is a source of variation in optimum swimming speed (U_{opt}), i.e., the speed that minimizes energy expenditure per unit of distance traveled.

Using gilthead sea bream (*Sparus aurata*), *E. lateralis* and Trinidadian guppy (*Poecilia reticulata*), we employed swimming respirometry and video analyses to test four hypotheses: (1) burst activity is an indicator of anaerobic power production and

correlates positively with the presence and magnitude of EPOC; (2) intraspecific diversity in MS or anaerobic capacity correlates positively with swimming performance; (3) there is a trade-off between U_{sus} and COT_{min} such that a high value of U_{sus} is associated with a high value of COT_{min} at the intraspecific level, and (4) variation in U_{sus} correlates positively with U_{opt}. Data on *S. aurata* were collected for the present study, whereas data on *E. lateralis* and *P. reticulata* were derived from previous studies (Svendsen et al., 2010, 2013).

MATERIALS AND METHODS

ANIMALS

A total of 13 gilthead sea bream (*Sparus aurata*) (body mass: 79.77 ± 2.38 g: standard length: 14.79 ± 0.24 cm (mean ± SE)) were obtained from a fish farm (Ferme Marine de Douhet) in France and kept in a flow-through holding tank (0.7 m^3) with saltwater (30%) at 10 ± 1°C at the University of Copenhagen in Denmark. *S. aurata* were fed daily with commercial trout pellets (Biomar, Brande, Denmark). All methods applied in the present study were in agreement with current Danish regulations for the treatment and welfare of experimental animals. No fish were used more than once, and there was no mortality during any of the tests.

RESPIROMETRY

A swimming respirometer (8.24 L) was used to measure oxygen consumption rate (MO_2; mg O_2 kg^{-1} h^{-1}) as a function of swimming speed (U). Water temperature inside the respirometer was maintained at 10.0°C (range: 9.9–10.1°C) using a temperature controlling instrument (TMP-REG; Loligo Systems; Tjele, Denmark). The respirometer was submerged in an ambient tank supplying water for the respirometer. Air stones maintained oxygen levels >95% air saturation in the ambient tank, and the water was recirculated through a loop consisting of a separate biological filter and a UV sterilizer (model UV-1000; Tetra Pond, Melle, Germany).

The swimming section of the respirometer was 32 × 9 × 11 cm (L × W × H). An impeller placed downstream of the swimming section was driven by an external electric motor that generated a re-circulating flow. Deflectors situated upstream of the swimming section collimated the flow. To promote rectilinear flow and a uniform velocity profile in the swimming section, water passed through an upstream honeycomb (7 mm cell diameter; Plascore Inc., Michigan, USA) producing a micro turbulent flow. A grid (10 mm) in the downstream direction bounded the swimming section. A vane wheel flow sensor (Höntzsch GmbH, Waiblingen, Germany) was used to measure water speeds in the swimming section. The measurements were used for a linear correlation between water speed and voltage output from the external motor controller.

Oxygen partial pressure (kPa) in the respirometer was measured using fiber optic sensor technology (PreSens, Regensburg, Germany). Intermittent flow respirometry was applied in accordance with previous studies (Steffensen, 1989). A computer-actuated pump was employed to replace water in the respirometer through a chimney as described previously (Svendsen et al., 2013). The software AutoResp (Loligo Systems Aps, Tjele, Denmark) was used to control the flush (240 s), wait (120 s) and measurement (540 s) phases. The settings provided one measurement of MO_2 per 15 min. The declining oxygen partial pressure (kPa) during the measurement phase was used to calculate MO_2 (mg O_2 kg^{-1} h^{-1}) using the equation:

$$MO_2 = \frac{K\ V\ \beta}{M} \quad (1)$$

where K is the linear rate of decline (kPa h^{-1}) in the oxygen content over time (h) in the respirometer, V is the volume of the respirometer (L) corrected for the volume of fish, β is the solubility of oxygen in the water (mg O_2 L^{-1} kPa^{-1}) ($\beta = 0.4480$) and M is the body mass of the fish (kg).

Preliminary trials demonstrated that the variation explained (R^2) by the linear equation fitted to the declining oxygen content (kPa h^{-1}), associated with each MO_2 measurement, was always ≥0.95, similar to previous studies (Claireaux et al., 2006; Svendsen et al., 2012). The oxygen content never fell below 17.6 kPa. Levels of background respiration (i.e., microbial respiration) were estimated from blank runs and used to correct MO_2 measurements (Jones et al., 2007; Svendsen et al., 2014).

BURST SWIMMING

Individual fish in the swimming section were recorded dorsally using a Hitachi video camera (model VM-H630E; Düsseldorf, Germany), situated above the swimming respirometer. A Pinnacle frame grabber (model PCTV USB2; Corel Corporation, Ontario, Canada) continuously transferred recordings to a PC, and fish 2D position (x, y coordinates) was tracked at 25 Hz using the software LoliTrack (Loligo Systems, Tjele, Denmark). A burst was defined as a forward excursion (≥ 4 cm) with the swimming speed increasing ≥ 5 cm s^{-1}. The number of bursts was determined over 3 min per respirometric loop (each 15 min) and used to estimate the total number of bursts per swimming speed (each 30 min; see below).

EXPERIMENTAL PROTOCOL

S. aurata for experiments were fasted for 48 h prior to respirometry to ensure a post-absorptive state. Fish mass (to nearest 0.01 g), length, depth and width (all to nearest 1 mm) were measured for pre-experimental calculation and correction of the solid blocking effects (Bell and Terhune, 1970; Gehrkel et al., 1990). Fish were acclimated to the respirometer for 12 h (overnight) while swimming at 0.5 body lengths per second (BL s^{-1}) prior to collection of data.

After the acclimation period, routine MO_2 ($MO_{2routine}$) was estimated as the average MO_2 during eight consecutive respirometric loops (i.e., 2 h) for each individual *S. aurata* swimming at 0.5 BL s^{-1} (i.e., acclimation speed) (Svendsen et al., 2010). At the individual level, the standard deviation (SD) of $MO_{2routine}$ was calculated using the eight MO_2 measurements. Next, *S. aurata* were exposed to progressive increments in the swimming speed of 0.5 BL s^{-1} every 30 min up to 2 BL s^{-1}. Using 30 min intervals for each swimming speed is a common approach (Schurmann and Steffensen, 1997; McKenzie et al., 2003, 2004; Lurman et al., 2007). Two measures of MO_2 were collected at each swimming speed. After completing measurements at 2 BL s^{-1}, *S. aurata* were exposed to speed increments of 0.25 BL s^{-1} every 30 min.

To examine the presence and magnitude of EPOC, the swimming speed was reduced to 0.5 BL s^{-1} (acclimation speed) after each exercise level from 2 BL s^{-1} and onwards. Specifically, detection of EPOC was carried out by comparing individual $MO_{2routine}$ + SD with the first post exercise MO_2 measurement during the 0.5 BL s^{-1} period that followed each new swimming exercise (Svendsen et al., 2010). It was considered evidence of EPOC if the first post exercise MO_2 was above $MO_{2routine}$ + SD. The measurements of MO_2 at 0.5 BL s^{-1} were continued until the MO_2 was below $MO_{2routine}$ + SD. When the MO_2 stabilized below $MO_{2routine}$ + SD, the swimming speed was increased to the next exercise level (i.e., the previous exercise speed + 0.25 BL s^{-1}). The protocol involving incrementally increasing swimming speeds followed by the procedure to detect EPOC was continued until fatigue.

DATA ACQUISITION AND ANALYSIS

Exercise MO_2 was recorded at increasing speeds from 0.5 BL s^{-1} to fatigue. Exercise MO_2 as a function of U in individual fish was described by the exponential equation:

$$MO_2 = a \exp(Ub) \quad (2)$$

where a is the MO_2 at zero speed ($U = 0$) and b is the rate of increase in the MO_2 as a function of U. The intercept with the y-axis (a) provides an estimate of the standard metabolic rate (MO_{2stand}) (Brett, 1964; Arnott et al., 2006; Svendsen et al., 2013). The analyses included a comparable data set on *P. reticulata* from an earlier study (Svendsen et al., 2013) in addition to the collected data on *S. aurata*. Following Svendsen et al. (2013), model fittings were limited to swim speeds without burst-assisted swimming. The analysis disregarded the measurements of post exercise MO_2 at 0.5 BL s^{-1} that were inserted to evaluate EPOC after swimming speeds ≥ 2 BL s^{-1}. Equation (2) was fitted to the individual data sets using mixed-effect models to account for temporal autocorrelation due to the repeated measurements. The analysis included an AR1 (autoregressive of order 1) covariance structure.

Maximum sustained (or aerobic) metabolic rate (MO_{2sus}) is defined as the maximum metabolic rate that can be maintained aerobically without the accumulation of anaerobic metabolic products that contribute to fatigue and negatively impact endurance (Hillman et al., 2014). In the present study, EPOC was detected when post exercise MO_2 was above $MO_{2routine}$ + SD, indicating anaerobic metabolism. At the individual level, MO_{2sus} was measured as the maximum recorded metabolic rate (over 0.5 h) at increasing swimming speeds without evidence of EPOC. The concurrent swimming speed was used as an estimate of the maximum sustained swimming speed (U_{sus}).

Active metabolic rate ($MO_{2active}$) was defined as the maximum exercise MO_2 that *S. aurata* maintained for 0.5 h without fatigue (Schurmann and Steffensen, 1997; Claireaux et al., 2005). Maximum metabolic rate (MO_{2max}) was defined as the highest exercise MO_2 measured during the complete swimming protocol (McKenzie et al., 2003; Svendsen et al., 2013; Binning et al., 2014). $MO_{2active}$ and MO_{2max} may be different, because $MO_{2active}$ is measured over 30 min, whereas MO_{2max} is often measured over a shorter period of time (minimum 15 min; one respirometric loop) and at a higher swim speed.

$MO_{2active}$ is usually assumed to be the maximum aerobic metabolic rate (Schurmann and Steffensen, 1997); however, to what extent $MO_{2active}$ includes an anaerobic component remains uncertain. If $MO_{2active}$ is the maximum aerobic metabolic rate, $MO_{2active}$ should not differ significantly from MO_{2sus}. To clarify differences between metabolic rates, a one way repeated measure ANOVA was used to compare MO_{2stand}, MO_{2sus}, $MO_{2active}$, and MO_{2max}. The test was followed by all pairwise comparison procedures (Holm-Šídák). The same test was employed to compare the swimming speeds associated with MO_{2sus}, $MO_{2active}$, and MO_{2max} (i.e., U_{sus}, U_{active}, and U_{max}).

The method described by Brett (1964) was used to calculate the critical swimming speed (U_{crit}). The protocol provides measurements that are repeatable in individual fish, suggesting that U_{crit} represent a measure of performance, which is a lasting characteristic of the organism (Claireaux et al., 2007; Oufiero and Garland, 2009).

The magnitude of EPOC (mg O_2 kg^{-1}) was quantified using protocols published previously (Svendsen et al., 2010). When EPOC was detected, the individual relationship between time t (h) and post exercise MO_2 was described using a double exponential equation:

$$MO_2 = a \exp(bt) + c \exp(dt) + MO_{2routine} \quad (3)$$

where a, b, c, and d are constants estimated using non-linear regression. Data included the exercise MO_2 at $t = 0$. The recovery period was terminated when the fitted curve intercepted $MO_{2routine}$ + SD and provided an estimate of recovery time (h). EPOC magnitude was calculated as the integrated area between the fitted curve (Equation 3) and $MO_{2routine}$ from $t = 0$ to the end of the recovery period. At the individual level, EPOC was combined with the exercise MO_2 to provide an estimate of the total cost of swimming (total MO_2; mg O_2 kg^{-1} h^{-1}), covering both aerobic and anaerobic components. The anaerobic capacity was estimated as the maximum EPOC observed in individual fish. Anaerobic capacity was quantified as mg O_2 kg^{-1} and mg O_2 kg^{-1} h^{-1}.

To test if the onset of burst swimming is a reliable predictor of the onset of EPOC, the minimum speed with burst swimming was correlated with the minimum speed with EPOC. The analysis was carried out using linear least square regression.

Linear mixed effects models were used to examine the relationship between the number of bursts and the magnitude of EPOC (mg O_2 kg^{-1}). Models included swimming speed as a covariate and interaction terms for swimming speed, burst number and fish identity. Temporal autocorrelation due to repeated measures was accounted for by including an AR1 covariance structure. The analysis included a comparable data set on *E. lateralis* from an earlier study (Svendsen et al., 2010).

The metabolic scope was calculated as MO_{2max}–MO_{2stand} in individual fish. The hypothesis that swimming performance (U_{crit}) is correlated with metabolic scope or anaerobic capacity in individual fish was tested using linear least square regression.

Cost of transport (COT) was calculated as mg O_2 kg^{-1} m^{-1} using the equation:

$$COT = \frac{MO_2}{U} \quad (4)$$

where MO_2 is the metabolic rate (mg O_2 kg^{-1} h^{-1}), and U is the corresponding swimming speed (m h^{-1}). The relationship between swimming speed and COT is usually U or ∪ shaped with high COT values at low and high swimming speeds (Rouleau et al., 2010).

For each individual fish, COT_{min} was measured using two different approaches: (A) COT_{min} was estimated as the lowest recorded value of COT. Following this approach, the optimum swimming speed (U_{opt}; the speed that minimizes energy expenditure per unit of distance traveled) was estimated as the swimming speed that corresponded to COT_{min}; (B) COT_{min} was estimated by first determining U_{opt} using the equation:

$$U_{opt} = \frac{1}{b} \quad (5)$$

where b originates from Equation (2) describing the individual relationship between swimming speed (cm s^{-1}) and MO_2 (mg O_2 kg^{-1} h^{-1}). Next, MO_2 at U_{opt} was calculated using Equation (2); and then COT_{min} was derived using Equation (4). Results from both approaches (A and B) to estimate COT_{min} and U_{opt} are reported, but figures are based on approach A. The analyses included a comparable data set on *P. reticulata* from an earlier study (Svendsen et al., 2013).

In a modeling study, Tokić and Yue (2012) presented evidence for a trade-off between U_{sus} and COT_{min}. The trade-off predicts a positive correlation between U_{sus} and COT_{min}, i.e., superior sustained swimming performance is associated with inferior swimming economy. To examine the trade-off in *S. aurata*, individual measures of U_{sus} and COT_{min} were correlated using linear least square regression. Similarly, this study tested for a relationship between U_{sus} and U_{opt} in individual fish. In addition to the data on *S. aurata*, the analyses of U_{sus}, COT_{min}, and U_{opt} included a comparable data set derived from an earlier study on *P. reticulata* (Svendsen et al., 2013).

Data were transformed [e.g., $\ln(x + 1)$] to meet the normality and homoscedasticity requirements of parametric analyses. The free statistical software R (R Development Core Team, 2014) and SigmaPlot (Systat Software, Erkrath, Germany) were used for statistical analyses and graphing. The R package nlme (Pinheiro et al., 2011) was employed to fit models. Results were considered significant at $P < 0.05$. All values are reported as means ± SE unless otherwise noted.

RESULTS

METABOLIC RATES AND SWIMMING PERFORMANCE

MO_{2stand}, MO_{2sus}, $MO_{2active}$, and MO_{2max} were measured at increasing speeds (**Figure 1**) and were all statistically different ($P < 0.05$). Notably, MO_{2sus} was lower than $MO_{2active}$, providing evidence of anaerobic metabolism (EPOC) in a significant number of *S. aurata* exercising at the level of $MO_{2active}$ (**Figure 1A**). The finding suggests that MO_{2sus} is a more appropriate measure of maximum sustained (or aerobic) metabolic rate than $MO_{2active}$. Similar to the metabolic values, the corresponding swimming speeds (U_{sus}, U_{active}, and U_{max}) differed significantly ($P < 0.05$) (**Figure 1B**). Interestingly, U_{sus} varied twofold between individuals with measurements ranging between 27 and 53.2 cm s^{-1}. Measures of U_{crit} were not included in **Figure 1**, but ranged between 35.3 and 56.5 cm s^{-1}, with an average value of 45.0 ± 1.6 cm s^{-1}. MO_{2sus} and U_{sus} corresponded to 79.3 ± 3.3% of MO_{2max} and 88.9 ± 1.9% of U_{crit}, respectively, with anaerobic metabolism detected above these exercise levels.

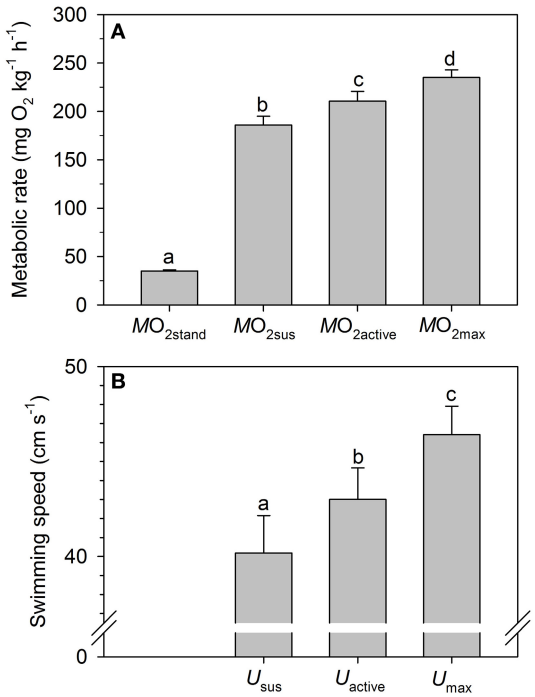

FIGURE 1 | Measurements of (A) metabolic rate (mg O_2 kg^{-1} h^{-1}) and (B) swimming speed (cm s^{-1}) in gilthead sea bream (*Sparus aurata*). Data include standard metabolic rate (MO_{2stand}), maximum sustained (or aerobic) metabolic rate (MO_{2sus}), active metabolic rate ($MO_{2active}$) and maximum metabolic rate (MO_{2max}). The measurements are defined in detail in the text. Corresponding swimming speeds **(B)** were derived from the metabolic measurements and include maximum sustained (U_{sus}), active (U_{active}) and maximum (U_{max}) swimming speeds. There are significant differences ($P < 0.05$) between the measurements of metabolism and swimming speed as indicated by the different letters.

EXERCISE MO_2 AND TOTAL MO_2 IN RELATION TO U_{crit}

EPOC was detected at all swimming speeds faster than U_{sus} and was combined with the exercise MO_2 to estimate the total MO_2. Because of the observed intraspecific variation in swimming performance, exercise MO_2 and total MO_2 were plotted as a function of $\%U_{crit}$ (**Figure 2A**) similar to previous studies (Lurman et al., 2007; Tudorache et al., 2008; Teulier et al., 2013). EPOC contributed to the total MO_2 starting at 86% of U_{crit} (**Figure 2A**). EPOC constituted 53.5 ± 4.9% of the total MO_2, ranging from 14.2 to 86.4% of total MO_2, at swimming speeds with evident EPOC. Thus, EPOC frequently constituted more than half of the swimming costs. Recovery time associated with EPOC lasted 7.8 ± 1.1 h, ranging from 1.0 to 20.9 h (**Figure 2B**).

POSITIVE CORRELATIONS BETWEEN BURST ACTIVITY AND ANAEROBIC METABOLISM (EPOC)

There was a positive linear relationship ($P < 0.0001$; $R^2 > 0.95$) between the minimum speed with EPOC and the minimum speed with burst swimming (**Figure 3**). The intercept with the y-axis was not significantly different from zero ($P > 0.65$). The relationship shows that the onset of burst swimming is a strong predictor of the onset of EPOC and anaerobic metabolism at increasing swimming speeds.

The relationship between the number of bursts and magnitude of EPOC was examined using a linear mixed effects model. The model included swimming speed as a covariate, but no significant effect ($P > 0.25$) or interactions ($P > 0.64$) related to swimming speed was detected. Model terms for swimming speed and interactions were therefore eliminated from further analyses. A comparable data set from a previous study on *E. lateralis* (Svendsen et al., 2010) was included in the analysis. For both data sets, the intercept with y-axis was not significantly different from zero ($P > 0.34$) and the slopes did not differ between the two data sets ($P > 0.94$). These findings indicated that the relationships between burst numbers and EPOC were similar in the two species, and the data were therefore, combined. The resulting common relationship (**Figure 4**) was described by the equation ($P < 0.0001$):

$$EPOC = 0.53\ (\pm 0.05)\ bursts \quad (6)$$

The relationship indicates that each burst corresponds to an average metabolic cost of 0.53 mg O_2 kg^{-1} (**Figure 4**).

NO CORRELATION BETWEEN U_{crit} AND METABOLIC SCOPE OR ANAEROBIC CAPACITY

Metabolic scope was estimated as MO_{2max}–MO_{2stand}, whereas anaerobic capacity was estimated as the maximum EPOC observed in individual fish. The maximum EPOC value was always associated with fish fatigue. There was no evidence that individual U_{crit} correlated with metabolic scope ($P > 0.87$; $R^2 <$

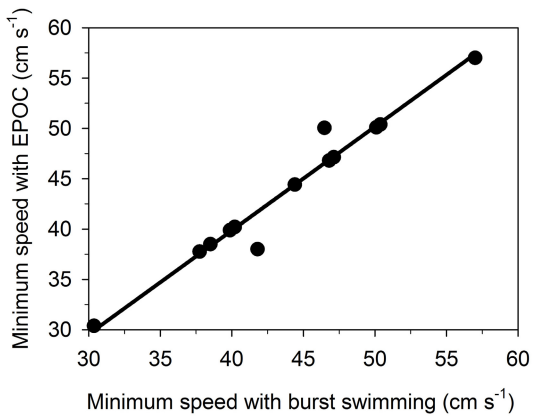

FIGURE 3 | The minimum speed with excess post exercise oxygen consumption (EPOC; mg O_2 kg^{-1}) correlates positively with the minimum speed with burst swimming ($P < 0.0001$; $R^2 > 0.95$) in gilthead sea bream (*Sparus aurata*). The relationship shows that the onset of burst swimming at increasing speeds indicates the onset of EPOC and therefore, anaerobic metabolism in individual fish.

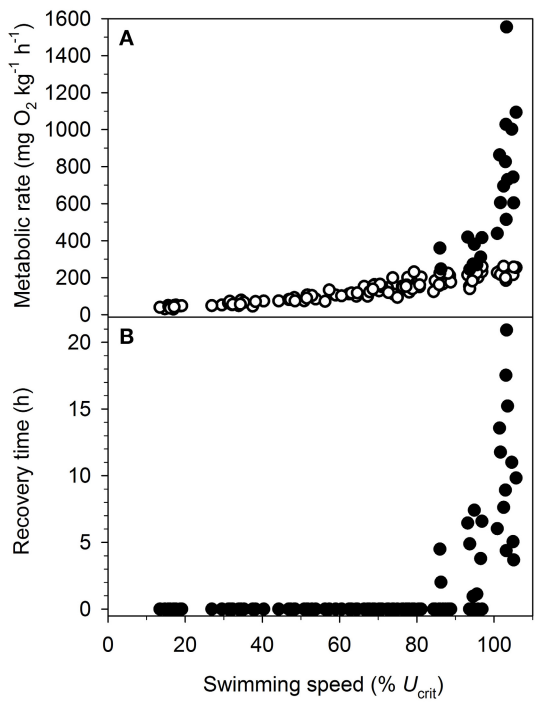

FIGURE 2 | (A) Metabolic rate (mg O_2 kg^{-1} h^{-1}) and **(B)** recovery time (h) in relation to swimming speed (% U_{crit}) in individual gilthead sea bream (*Sparus aurata*). U_{crit} is the critical swimming speed and defined in detail in the text. For **(A)**, metabolic rates were measured in the swimming fish (exercise MO_2; open symbols) and as excess post exercise oxygen consumption (EPOC; mg O_2 kg^{-1}). Exercise MO_2 and EPOC were combined to estimate the total metabolic cost of swimming (total MO_2; closed symbols). For **(B)**, recovery time (h) reflects the duration of EPOC after single swimming exercises (up to 30 min).

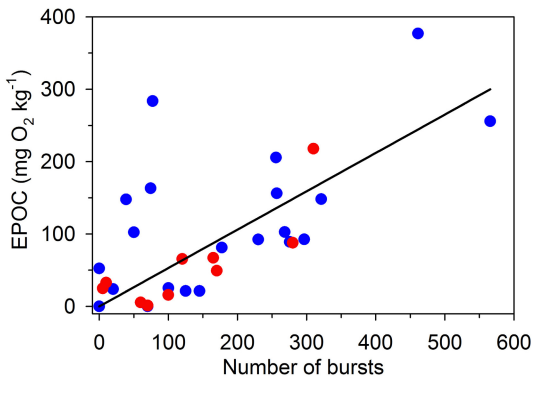

FIGURE 4 | The magnitude of excess post exercise oxygen consumption (EPOC; mg O_2 kg^{-1}) correlates positively with burst activity in gilthead sea bream (*Sparus aurata*) (blue symbols) and striped surfperch (*Embiotoca lateralis*) (red symbols). Data on *S. aurata* are from the present study, whereas data on *E. lateralis* are from Svendsen et al. (2010). The linear fit ($P < 0.0001$) reflects the pooled data set for both species (Equation 6), because species-specific regression slopes and intercepts with the y-axes are not statistically different ($P > 0.34$). The relationship suggests that a burst represents a metabolic cost of 0.53 mg O_2 kg^{-1}.

0.01) or with anaerobic capacity ($P > 0.57$; $R^2 < 0.04$) (data not shown). The analyses of anaerobic capacity involved maximum EPOC quantified as mg O_2 kg^{-1} and mg O_2 kg^{-1} h^{-1}.

NO TRADE-OFF BETWEEN U_{sus} AND COT_{min}

This study examined a trade-off between U_{sus} and COT_{min} by comparing swimming performance and metabolism in *S. aurata* and *P. reticulata*. In terms of *S. aurata*, U_{sus} was assumed to correspond to the highest swimming speed without EPOC (**Figures 1**, **2**). Data on *P. reticulata* were derived from Svendsen et al. (2013). While EPOC was not measured in *P. reticulata*, the study quantified burst activity in individual *P. reticulata* at increasing speeds. Using the relationship between the onset of burst swimming and the onset of EPOC (**Figure 3**), EPOC occurrence at increasing speeds, and thereby U_{sus}, were estimated in individual *P. reticulata*. COT_{min} in *P. reticulata* was estimated in the same fashions (approaches A and B) as in *S. aurata* (Equations 4 and 5). The relationships between U_{sus} and COT_{min} were examined using linear least square regressions (**Figure 5**). For both species, there was no evidence of a trade-off between U_{sus} and COT_{min}. In fact, there were significant negative correlations between U_{sus} and COT_{min}, revealing that individuals exhibiting superior sustained swimming performance (i.e., high U_{sus}) also exhibit superior swimming economy (i.e., low COT_{min}) (**Figure 5**). The negative correlations between U_{sus} and COT_{min} were evident in both species and regardless of the approach (A and B) used to estimate COT_{min} (all $P < 0.005$; $R^2 > 0.53$). Data in **Figure 5** are based on approach A.

POSITIVE CORRELATIONS BETWEEN U_{sus} AND U_{opt}

There were significant positive correlations between U_{sus} and U_{opt} (**Figure 6**). The analyses included data on *S. aurata* (**Figure 6A**) and *P. reticulata* (**Figure 6B**) and revealed that individuals exhibiting superior sustained swimming performance (i.e., high U_{sus}) also exhibit superior optimum swim speed (i.e., high U_{opt}). The positive correlations between U_{sus} and U_{opt} were evident in both species and regardless of the approach used to estimate U_{opt} (approach A: all $P < 0.005$; $R^2 > 0.40$; approach B: all $P < 0.05$; $R^2 > 0.26$). Data in **Figure 6** are based on approach A.

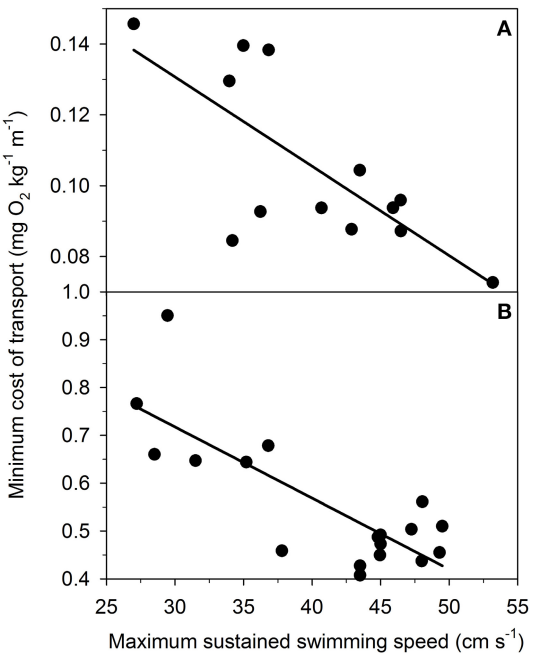

FIGURE 5 | Minimum cost of transport (COT_{min}; mg O_2 kg^{-1} m^{-1}) correlates negatively with maximum sustained swimming speed (U_{sus}; cm s^{-1}) in (A) gilthead sea bream (*Sparus aurata*; $n = 13$) and (B) Trinidadian guppy (*Poecilia reticulata*; $n = 18$). Data on *S. aurata* are from the present study, whereas data on *P. reticulata* are derived from Svendsen et al. (2013). Both relationships are statistically significant ($P < 0.005$; $R^2 > 0.53$). The relationships suggest that superior sustained swimming performance (i.e., high U_{sus}) is associated with superior swimming economy (i.e., low COT_{min}) in both species. Note that y-axes differ between the two panels.

DISCUSSION

This study demonstrated the energetic importance of anaerobic metabolism during unsteady locomotion. There was no evidence of U_{crit} correlating with MS or anaerobic capacity. Moreover, we provided intraspecific evidence that a high U_{sus} is coupled with low COT_{min} and high U_{opt} in individual fish. Specifically, our results reveal that burst swimming is associated with anaerobic metabolism and a substantial metabolic cost, which is expressed as EPOC. Our intraspecific results on two teleost species are at odds with the conjecture that there is a trade-off between U_{sus} and COT_{min} as indicated by Tokić and Yue (2012). By applying the trade-off, the authors provided a model that explained variation in morphology in various teleost and cetacean species. In contrast, the present study is based on intraspecific data collected empirically. Our findings suggest that intraspecific variation in U_{sus} and COT_{min} is not driven by a trade-off producing a high U_{sus} in some individuals and a low COT_{min} in other individuals. Because the results suggest that U_{sus} and COT_{min} are optimized concurrently, it is unlikely that the trade-off drives intraspecific morphological variation.

Previous studies have demonstrated that the U_{crit} protocol includes swimming powered by both aerobic and anaerobic metabolism (Burgetz et al., 1998; Richards et al., 2002). In *E. lateralis*, EPOC and anaerobic metabolism is present at 88% of U_{crit} (Svendsen et al., 2010). Corroborating previous results, the present study found evidence of EPOC starting at swimming speeds corresponding to 86% of U_{crit}. At higher speeds, EPOC increased rapidly and constituted up to 86% of the total MO_2. The maximum value of EPOC was always associated with fatigue. Likewise, beginning at 89% of U_{crit} in Atlantic cod (*Gadus morhua*), Lurman et al. (2007) found evidence of anaerobic metabolism as indicated by decreasing levels of phosphocreatine and intracellular pH and increasing levels of inorganic phosphate. Our study corroborates that the U_{crit} protocol involves depletion of both aerobic and anaerobic resources, and shows that the metabolic costs associated with the recovery from the anaerobic perturbation (i.e., EPOC) may constitute the majority of the swimming costs. The results highlight the importance of measuring both exercise MO_2 and EPOC to estimate the total metabolic costs of swimming in fish approaching prolonged and burst

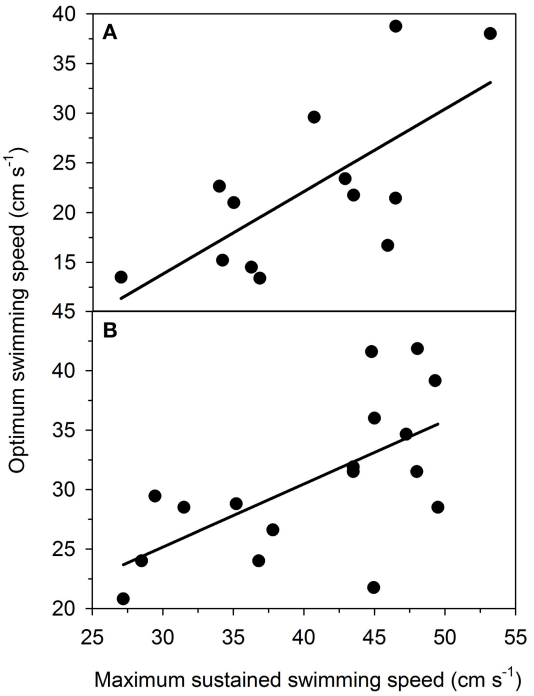

FIGURE 6 | Optimum swimming speed (U_{opt}; cm s^{-1}) correlates positively with maximum sustained swimming speed (U_{sus}; cm s^{-1}) in (A) gilthead sea bream (*Sparus aurata*; $n = 13$) and (B) Trinidadian guppy (*Poecilia reticulata*; $n = 18$). Data on *S. aurata* are from the present study, whereas data on *P. reticulata* are derived from Svendsen et al. (2013). Both relationships are statistically significant ($P < 0.005$; $R^2 > 0.40$). The relationships suggest that superior sustained swimming performance (i.e., high U_{sus}) is associated with superior optimum swimming speed (i.e., high U_{opt}) in both species. Note that y-axes differ between the two panels.

swimming speeds. In the absence of EPOC measurements, the metabolic cost of swimming may be significantly underestimated.

This study shows that the onset of burst-assisted swimming is closely related to the onset of EPOC at increasing swimming speeds in individual fish. The initiation of burst swimming is therefore a strong predictor of EPOC and anaerobic metabolism. Similarly, we found that the magnitude of EPOC increases linearly with the number of bursts. The present data are consistent with a previous study on *E. lateralis* (Svendsen et al., 2010). Combining the two data sets suggests that each burst corresponds to an energetic cost of 0.53 mg O_2 kg^{-1}. *E. lateralis* is a labriform swimmer (i.e., pectoral fins used for propulsion at low and medium swimming speeds) whereas *S. aurata* is an axial swimmer (i.e., axial undulation used for propulsion). The fact that we found no differences in the two relationships between bursts and EPOC indicates that the metabolic cost of burst swimming may be similar across fish species employing disparate types of locomotion.

MS is predicted to play a major role in relation to effects of climate change, and other anthropogenic stressors including hypoxia, on aquatic exothermic animals (Claireaux and Lefrançois, 2007; Chabot and Claireaux, 2008; Guderley and Pörtner, 2010; Pörtner, 2010; Pörtner and Peck, 2010; McBryan et al., 2013; Seth et al., 2013; Di Santo, 2015). It remains uncertain, however, to what extent intraspecific diversity in MS varies with other important physiological traits including locomotor performance. The present study measured intraspecific variation in MS and anaerobic capacity (i.e., maximal EPOC) in *S. aurata* and correlated data with individual variation in swimming performance (U_{crit}). We found no evidence that diversity in MS or anaerobic capacity correlates with U_{crit} ($P > 0.57$) indicating that other factors, including morphological (Rouleau et al., 2010) or biomechanical (Svendsen et al., 2013) traits, drive the variation in swimming performance.

It is possible that the lacking relationship between MS and U_{crit} was caused by our method of measuring MO_{2max}. Similar to previous studies (McKenzie et al., 2003; Svendsen et al., 2013; Binning et al., 2014), we used an U_{crit} protocol to measure MO_{2max} involving progressive increments in the swimming speed of 0.25 BL s^{-1} every 30 min, starting from 2 BL s^{-1} and until fatigue. Our protocol differed, however, from conventional protocols, because we inserted periods with swimming speeds adjusted to 0.5 BL s^{-1} (acclimation speed) for measurements of EPOC after each swimming speed $\geq$ 2 BL s^{-1}. Although the mechanistic basis is unknown, it is possible that our protocol affected the measurements of MO_{2max}. As an alternative to the U_{crit} protocol, a number of recent studies have used a chase protocol to measure MO_{2max} (Norin and Malte, 2011, 2012; Svendsen et al., 2014). The U_{crit} protocol is often assumed to provide measures of MO_{2max} (Farrell and Steffensen, 1987; Hammer, 1995) and may in fact elicit values of MO_{2max} that are higher than the values elicited by the chase protocol (Roche et al., 2013). Therefore, it is unlikely that a significant relationship between MS and swimming performance would have been revealed if we had used a chase protocol instead of the U_{crit} protocol to measure MO_{2max}. In humans, MO_{2max} is typically measured using test protocols that are much faster (Barker et al., 2011; Vanhatalo et al., 2011; Mauger et al., 2013) than the U_{crit} protocol used in the present study. While a protocol that continuously steps up the swimming speed in much faster pace than the U_{crit} protocol might produce higher values of MO_{2max} (and therefore MS) and swimming performance (Farrell, 2008), it remains to be tested if the methodology would produce a significant relationship between MS and swimming performance. A faster protocol would rely more on anaerobic metabolism to power swimming (Farrell, 2008; Poulsen et al., 2012), and so a relationship between anaerobic capacity and swimming performance might be revealed.

A recent study emphasized a trade-off between U_{sus} and COT_{min} driving morphological diversity in aquatic locomotion (Tokić and Yue, 2012). The trade-off assumes constraints in optimizing U_{sus} and COT_{min} simultaneously, suggesting that aquatic species may optimize either U_{sus} or COT_{min}. By applying the trade-off, Tokić and Yue (2012) modeled morphological variation and reported congruent morphological variation in several extant aquatic species. The present study examined the trade-off within two teleost species and found no support for the trade-off. In fact, data revealed a significant negative correlation between U_{sus} and COT_{min}, suggesting that individuals with high U_{sus} also exhibit low COT_{min}. The negative relationship indicates that the two traits are optimized simultaneously and could be related to the same mechanistic basis without constraints. Interestingly, studies

are increasingly uncovering significant intraspecific variation in locomotor performance and metabolic rate (Nelson et al., 2003; Langerhans, 2008, 2009a; Dalziel et al., 2011, 2012; Dalziel and Schulte, 2012; Svendsen et al., 2013; Binning et al., 2014). The present study indicates that intraspecific morphological variation, associated with intraspecific variation in locomotor performance and metabolic rate, is not driven by a trade-off between U_{sus} and COT_{min}.

There are a number of reasons why we may not observe a trade-off between U_{sus} and COT_{min} in our intraspecific data. Variation between species is much more pronounced than between individuals of the same species. Therefore, interspecific variation may better reflect the full spectrum of functional trade-offs that influences morphological variation related to aquatic locomotion. It is also possible that a trade-off between U_{sus} and COT_{min} is present in the two tested fish species, but not expressed at the whole-organism level, because of compensating or masking factors involving morphological, physiological and/or biomechanical traits. Moreover, our estimates of U_{sus} and COT_{min} based on respirometry and video analysis might be misleading. For example, it is possible that estimates of U_{sus} using measures of EPOC (*S. aurata*) and burst-assisted swimming (*P. reticulata*) do not accurately reflect maximum sustained swimming speeds. MO_2 is, however, a well-established proxy for aerobic metabolic rate, and the gait transition from steady to unsteady (i.e., burst-assisted) swimming is a well-known indicator of the shift from aerobic to anaerobic power production (Peake and Farrell, 2004, 2006; Peake, 2008; Svendsen et al., 2010). Similarly, it is possible that the use of forced linear swimming to estimate U_{sus} and COT_{min} provides results that do not necessarily reflect natural conditions, because fish typically swim spontaneously in a non-linear fashion with the relationship between swimming speed and metabolic rate differing from linear swimming (Steinhausen et al., 2010).

Diversity in locomotor performance and metabolism can be important sources of variation in animal behaviors. For example, Hillman et al. (2014) suggested that variation in physiological capacity for movement influences dispersal and therefore fine-scale genetic structure of several vertebrate groups. At the intraspecific level, physiological performance is an important determinant of behaviors related to schooling (Killen et al., 2011), territory acquisition and defense and foraging (Breau et al., 2011; Killen et al., 2014). Likewise, physiological and energetic states may influence behaviors in migratory species (Poulsen et al., 2010; Boel et al., 2014). Recent studies have shown that exercise training that increases swimming performance may change the behavior of animals and cause elevated boldness and exploratory tendency (Sinclair et al., 2014). The mechanistic basis of the relationship between exercise training and behavior could be related to the positive relationship between U_{sus} and U_{opt} found in the present study. Because exercise training increases aerobic potentials in red and white musculature (Davison, 1997) and swimming performance (Farrell et al., 1990; Sinclair et al., 2014), exercise training should also elevate U_{sus} and therefore U_{opt}. Typically, fish swim spontaneously at speeds corresponding to U_{opt} (Videler, 1993; Tudorache et al., 2011). This hypothesis suggests that exercise training increases spontaneous swimming speeds via the positive relationship between U_{sus} and U_{opt}. It seems likely that increased spontaneous swimming speed is associated with elevated boldness and exploratory tendency as observed by Sinclair et al. (2014). Therefore, the positive relationship between U_{sus} and U_{opt} could provide a mechanistic link between physiological and behavioral phenotypes. Nevertheless, this hypothetical framework warrants additional study to clarify the mechanistic basis of intraspecific correlations between physiological and behavioral phenotypes.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: BT, JCS, JFS. Performed the experiments: BT. Analyzed the data: JCS, GAC, BT. Contributed reagents/materials/analysis tools: JFS. Wrote the paper: JCS. Revised the manuscript critically for important intellectual content: JCS, BT, GAC, JFS.

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The interplay between aerobic metabolism and antipredator performance: vigilance is related to recovery rate after exercise

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When attacked by a predator, fish respond with a sudden fast-start motion away from the threat. Although this anaerobically-powered swimming necessitates a recovery phase which is fueled aerobically, little is known about links between escape performance and aerobic traits such as aerobic scope (AS) or recovery time after exhaustive exercise. Slower recovery ability or a reduced AS could make some individuals less likely to engage in a fast-start response or display reduced performance. Conversely, increased vigilance in some individuals could permit faster responses to an attack but also increase energy demand and prolong recovery after anaerobic exercise. We examined how AS and the ability to recover from anaerobic exercise relates to differences in fast-start escape performance in juvenile golden gray mullet at different acclimation temperatures. Individuals were acclimated to either 18, 22, or 26°C, then measured for standard and maximal metabolic rates and AS using intermittent flow respirometry. Anaerobic capacity and the time taken to recover after exercise were also assessed. Each fish was also filmed during a simulated attack to determine response latency, maximum speed and acceleration, and turning rate displayed during the escape response. Across temperatures, individuals with shorter response latencies during a simulated attack are those with the longest recovery time after exhaustive anaerobic exercise. Because a short response latency implies high preparedness to escape, these results highlight the trade-off between the increased vigilance and metabolic demand, which leads to longer recovery times in fast reactors. These results improve our understanding of the intrinsic physiological traits that generate inter-individual variability in escape ability, and emphasize that a full appreciation of trade-offs associated with predator avoidance and energy balance must include energetic costs associated with vigilance and recovery from anaerobic exercise.

Keywords: metabolic rate, aerobic scope, predator–prey interactions, swimming, teleost fish

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Introduction

Numerous mechanisms have evolved that allow animals to escape predators at virtually all points along the sequence of a typical predator–prey encounter (Hart, 1997; Killen, 2011). For fish,

a fast-start escape response occurs during the critical first few milliseconds of a predator attack. It is a brief, sudden, and anaerobically-powered acceleration by the prey, consisting of a unilateral muscle contraction in a direction opposite to the threat, followed by an additional contraction on the other side of the body to propel the fish further away from the predator (Domenici and Blake, 1997). Escape responses in fish are usually controlled by a pair of giant reticulospinal neurons in the hindbrain, the Mauthner cells (Eaton et al., 1977), although non-Mauthner cell responses are also possible (Kohashi and Oda, 2008). Such non-Mauthner escape responses, however, tend to show longer latencies between stimulus detection and the resulting escape attempt (Liu and Fetcho, 1999; Eaton et al., 2001; Kohashi and Oda, 2008). Responsiveness and locomotory performance during this response likely influences which individuals are capable of surviving a predator attack (Domenici, 2010), and shows repeatable variation among individuals of the same species (Marras et al., 2011). However, the factors that generate and maintain this variation remain largely unknown.

The ideal strategy for avoiding predators is a balance between fleeing too early, which can result in lost foraging opportunities, and fleeing too late, which can increase the risk of being captured (Ydenberg and Dill, 1986; Krause and Godin, 1996; Bohórquez-Herrera et al., 2013). The optimal position of this trade-off may vary among individuals in relation to intrinsic traits (Jones and Godin, 2010). For example, individual fish vary in the rate at which they physiologically recover from anaerobic exercise such as that used during fast-starts (Marras et al., 2010; Killen et al., 2014). The waste products from this process must subsequently be metabolized during a recovery phase which is fueled aerobically (Richards et al., 2002). This post-exercise rise in aerobic metabolism represents the energetic cost of the escape response, but will also occupy a proportion of the individual's aerobic scope (AS) until recovery is complete, possibly constraining performance of other oxygen-consuming physiological functions. Furthermore, although escape responses are fueled anaerobically, they are negatively affected by hypoxia (Domenici et al., 2007), suggesting that escape responses and oxygen needs are not completely decoupled. Overall, variation in recovery rate after anaerobic exercise among individual fish could affect the relative costs and benefits of engaging in a fast-start response. Fish that recover more slowly from anaerobic exercise may incur a greater realized cost when engaging in a fast-start escape response, particularly if they have a reduced capacity for locomotory performance or other aerobic physiological processes during recovery. They may therefore be more reluctant to react to a potential attack or react more slowly. Further, fish with a higher AS are able to recover from anaerobic exercise faster (Marras et al., 2010; Killen et al., 2014), and so it is possible that fish with a larger AS may be more able to incur the costs associated with recovery, and therefore show greater responsiveness or performance during an escape response. Conversely, it is also possible that fish that react sooner or move faster during an attack may show a slower rate of recovery after anaerobic exercise, if their recovery is delayed due to the energetic costs of vigilance. On a mass-specific basis, the brain is one of the most metabolically active organs in the body (Rolfe and Brown, 1997), and high alertness and preparedness to escape may therefore entail an increased energetic demand (Millidine et al., 2006). There may also be costs associated with the maintenance of sensory systems that allow for increased threat perception and responsiveness. Overall, any such increases in energetic demand could prolong the phase of physiological recovery after an escape attempt or any other form of anaerobic metabolism.

Environmental factors can also affect locomotory performance, and often, the degree of variation in performance traits observed among individuals (Killen et al., 2013). Temperature, for example, has a strong influence on the metabolic physiology and locomotory performance of ectotherms (Angilletta et al., 2002) and neural performance (Montgomery and Macdonald, 1990). The effect of temperature on the fast-start response of fish is variable among species (Wilson et al., 2010), but is known to affect escape responsiveness (Webb, 1978; Preuss and Faber, 2003). Response latency tends to decrease at higher temperatures as a result of the effect of temperature on the speed of nerve conduction. However, fish subject to acute high temperature treatments may increase their latency as found in heat shock experiments in which high temperatures yielded longer reaction distances (Webb and Zhang, 1994). An increase in temperature has been associated with an increase maximum velocity during the fast-start response (Beddow et al., 1995) as a result of an increase muscle power output (Wakeling, 2006), but there appears to be a threshold temperature beyond which performance declines (Johnston and Temple, 2002). Furthermore, thermal acclimation can result in some degree of compensation (Johnson and Bennett, 1995; Wakeling, 2006). Temperature also influences the degree of physiological stress incurred during anaerobic exercise and the rate of subsequent recovery (Suski et al., 2006). Finally, temperature has a profound effect on AS in fishes, with AS being highest at a species-specific optimum temperature and then reduced at temperature above or below this point (Claireaux and Lefrancois, 2007; Pörtner and Farrell, 2008). It is therefore possible that temperature could modulate any effects of physiological traits on the fast-start escape response.

In this study we examined how the ability to recover from anaerobic exercise relates to differences in fast-start escape performance among individual fish. Further, we examined how acclimation temperature might modulate such relationships. We studied these issues in juvenile golden gray mullet *Liza aurata*. The young of this species inhabits lagoon environments where they are frequently targeted by a range of piscine and avian predators. Specifically, we investigated two alternative hypotheses regarding the interplay between aerobic metabolism and the fast-start escape response: (1) The aerobic-scope driven hypothesis: that individuals which respond to a simulated attack sooner and with a higher level of performance are those that recover sooner after anaerobic exercise, or that have a higher AS (i.e., high AS allows for a low threshold for escape); and (2) The vigilance-driven hypothesis: that fish with fast reaction times and high escape performance are those that show longer recovery rates, because they are the most vigilant and therefore have higher energetic demands that prolong physiological recovery after anaerobic exercise. The results of this study will improve our understanding of how intrinsic traits and environmental factors interact to affect anti-predator behaviors, and specifically

performance during escapes, among individual animals of the same species.

Materials and Methods

Animals

Juvenile Golden gray mullet ($n = 42$) were captured from the wild (Stagno di Cabras, Sardinia, Italy) in January 2012. Once at the laboratory (IAMC-CNR, Oristano, Sardinia, Italy) they were held under a 12–12 h light/dark photoperiod in a large cylindrical tank (2 m diameter, 1.5 m water depth) supplied with re-circulating, filtered natural seawater at a constant temperature (20°C) for 2 weeks. After this period, three groups of 14 individuals each were transferred to circular 100-L tanks, whilst initially retaining environmental parameters. Final acclimation temperatures of either 18, 22, or 26°C were reached by changing the water temperature by 1°C per day. Fish were left 1 month at the final acclimation temperature before being used in the experiment. Fish in the holding tanks were fed daily with a maintenance ration consisting of dried feed pellets. Individuals were fasted for 36 h before use in experiments. At experimentation, fish were 12.8 ± 0.81 cm total length and 15.3 ± 0.48 g mass (mean ± S.E.).

The fish were held, and the non-lethal experiments were conducted, in accordance with the laws governing animal experimentation in Italy. The IAMC-CNR facility of Oristano, where the fish were held and the experiments performed, is recognized by the Italian Government as a certified facility for fish rearing and ecophysiological experimentation (D.lgs. 116/92, Decreto n° 136/2011-A).

Experimental Protocol

As described and defined below, all traits were measured on every individual. This includes the following metabolic traits: standard metabolic rate (SMR), routine metabolic rate (RMR), maximal metabolic rate (MMR), aerobic scope (AS), excess post-exercise oxygen consumption (EPOC), and recovery time (T_R) after exhaustive exercise; and the fast-start components: response latency, maximum speed (U_{max}), maximum acceleration (A_{max}), and turning rate.

Fish were first transferred to a fast-start arena, and left undisturbed for 12 h before testing for the escape response (see below for details). At the end of the escape response test fish were removed from the escape test arena and inserted into a static respirometer to determine SMR and RMR. The following day, aerobic scope (AS; i.e., the capacity to supply oxygen for all aerobic tasks above maintenance, including swimming, digestion, and recovery from anaerobic exercise) was determined by obtaining the difference between MMR and SMR.

Fast-start Test

The experimental set-up comprised of a circular tank (100 cm diameter × 80 cm depth and 25 cm water depth), supplied with re-circulating seawater at the fish acclimation temperature. The escape response of the fish was induced by mechanical stimulation (Dadda et al., 2010). The stimulus was a PVC cylinder with a tapered downward end and an iron bolt at the opposite end (10 cm height, 2 cm diameter, and weighing 35 g). The stimulus was released by an electromagnet from a height of 150 cm above the water surface. To prevent visual stimulation before contact with the water surface, the stimulus was released into a vertical PVC tube (15 cm of diameter) ending 0.5 cm before the water surface. A mirror inclined at 45° was used to identify the exact time the stimulus entered into the water. Light was supplied by two 250 W spotlights. The escape response arena was covered by a black tarpaulin, to screen the fish from visual disturbance. A high speed camera (Casio EXILIM High Speed EX-FH100, Casio Computer Company Ltd., Japan) was positioned above the experimental tank and recorded the escape response at 240 Hz. The fish were startled only within a range of angles between 80° and 100° relative to the stimulus, and at a relatively fixed distance from the stimulus, of between 20 and 30 cm.

The following variables were analyzed according to Marras et al. (2011): (1) Responsiveness, i.e., the percentage of fish, of the total analyzed, that responded to the stimulation with an escape response after being stimulated; (2) Latency, defined as the time interval between when the stimulus broke the water surface and the first detectable escape movement of the fish; (3) Distance-time variables, evaluated within a fixed time (58 ms; Dadda et al., 2010) which approximately corresponded to the average duration of stage 1 and 2 of the fast start response of all fish considered for all tests (mean escape duration), including cumulative distance, U_{max}, and A_{max}; (4) Stage 1 turning rate, calculated as the angle between the segment joining the center of mass and the tip of the head, at the beginning and at the end of the stage 1, divided by the stage duration. A polynomial regression procedure with five smoothed moving points was then applied for each derivative procedure (i.e., speed and acceleration) as described by Lanczos (1956).

Respirometry

Fish were removed from the escape response arena and placed in static respirometers (0.5 L, Loligo Systems, Denmark) immersed in an outer tank maintained at either 18, 22, or 26°C (as appropriate depending on the acclimation temperature for each fish. Instantaneous oxygen uptake (MO_2, in mg O_2 h^{-1}) was measured by intermittent flow respirometry (Steffensen, 1989) once every 30 min. Temperature was kept constant for the duration of the experiment. Water flow from the external bath through the respirometers was driven by an external pump that was set to turn on and off for alternating 15-min periods. This allowed decreases in water oxygen content to be measured every 15 s for 15 min while the respirometer was in the closed state. The respirometer was then flushed with aerated water for 15 min. The oxygen consumption during each closed phase was calculated using linear least squares regression (excluding the first and last 2 min of each closed phase). Water oxygen levels were measured with optodes (Oxy-4 mini; PreSens Precision Sensing GmbH, Regensburg, Germany) and associated software (Pre-Sens Oxy 4v2). Fish remained in the chambers for approximately 24 h. Whole-animal SMR (in mg O_2 h^{-1}) was estimated as the lowest tenth percentile of measurements taken throughout the measurement period (Dupont-Prinet et al., 2010; Killen, 2014), excluding the first 2 h of confinement in the chambers during which oxygen consumption was often elevated. RMR was measured as the mean

level of oxygen uptake during this time. Fish were then removed from the chambers, placed into a circular arena (100 cm diameter × 80 cm depth and 25 cm water depth) and manually chased until exhaustion until they were no longer responsive (Clark et al., 2013; Killen, 2014). They were then placed back into the respirometry chambers, and MO_2 was measured as previously described for 5 h. Measurements of background microbial respiration in the system were taken before and after the oxygen measurement in the respirometers. The highest rate of MO_2 measured during the 5 h period after exhaustive exercise was taken as the MMR of the fish, and almost always occurred during the first measurement period after exercise. This method for determining MMR assumes that maximal rates of oxygen uptake are achieved during the recovery from the bout of exhaustive anaerobic exercise (Reidy et al., 2000; Killen et al., 2007; Clark et al., 2013). Aerobic scope was calculated as the difference between MMR and SMR. Individual recovery time (T_R) was assessed as the time required for oxygen uptake to return from the maximal post exercise MO_2 to the level at which the animal had available 50% of its total AS (Marras et al., 2010; Killen et al., 2014). EPOC for each individual was estimated by calculating the area under the sixth-order polynomial recovery function, above RMR, until the time at which fitted values were equal to individual RMR (Killen et al., 2014). For some fish (12/42 fish, with only one fish predicted to take longer than 7 h to return to RMR), values never returned to RMR within the 5 h measurement period after exercise. In these cases EPOC was estimated using an extrapolation of the recovery function until the fitted values were equal to RMR. EPOC represents the increase in oxygen consumption above routine levels occurring during recovery from a bout of exhaustive anaerobic exercise and represents the anaerobic capacity of an animal (Gastin, 1994; Lee et al., 2003). Note that fish were always transferred between experimental setups without air exposure. At the end of the metabolic measurements, fish were removed from the respirometers and measured for total length and mass.

Data and Statistical Analysis

All tests were performed using SPSS Statistics (v. 20). The effect of temperature on metabolic traits (i.e., SMR, RMR, MMR, AS, EPOC, and recovery time) was assessed using general linear models. Absolute vales for either SMR, RMR, MMR, AS, EPOC, or T_R were used as the dependent variable, with temperature as a categorical explanatory variable and body mass as a covariate. We then examined links among metabolic traits, temperature, and fast-start components using general linear models. In a given model, fast-start components (either of response latency, U_{max}, A_{max}, turning rate) were used as the dependent variable, and SMR, AS, EPOC, T_R, body mass and temperature were included as explanatory variables. Interactions between each explanatory variable and temperature were removed when not significant and the models re-run.

Results

Only four out of 42 fish were not responsive to the stimulus designed to elicit a fast-start escape response. Although all four non-responsive fish were among those acclimated to 26°C, the small number of fish that were non-responsive precluded statistical analyses of the factors affecting responsiveness.

Fish acclimated to warmer temperatures had a higher RMR [**Figure 1**; GLM, effect of temperature, $F_{(2,42)} = 5.624$, $p = 0.007$], but none of SMR, MMR, AS, EPOC, or T_R were influenced by acclimation temperature (**Figure 1**; GLM, effect of temperature, $p > 0.05$ in all cases).

Models examining the simultaneous influence of temperature and metabolic traits on fast-start components showed that fish with a longer T_R showed shorter response latencies [**Figure 2**; **Table 1**; GLM, effect of T_R, $F_{(1,37)} = 7.064$, $p = 0.012$]. Fish with

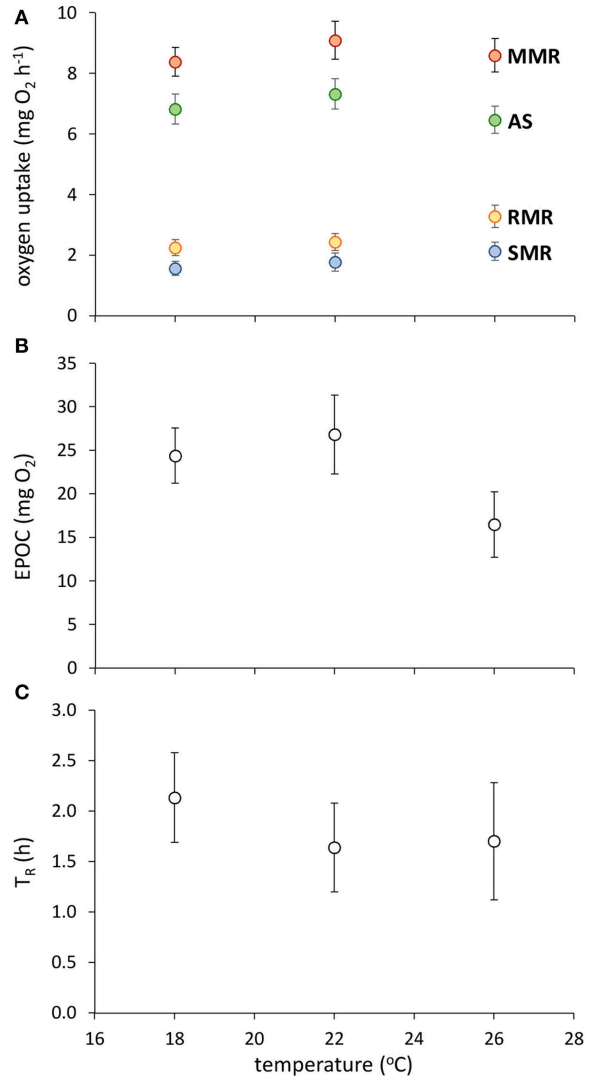

FIGURE 1 | Effect of temperature on metabolic traits in juvenile golden gray mullet. (A) Traits associated with aerobic metabolism (SMR, standard metabolic rate; RMR, routine metabolic rate; AS, aerobic scope; MMR, maximal metabolic rate); **(B)** anaerobic capacity, as indicated by excess post-exercise oxygen consumption (EPOC); and **(C)** recovery rate after exhaustive exercise, as indicated by the time taken until recovery of 50% of total aerobic scope (T_R). There was no significant effect of temperature on any response variable (see Results). Error bars = s.e.m; $n = 14$ fish per temperature.

a higher AS also had shorter response latencies [**Table 1**; GLM, effect of AS, $F_{(1,38)} = 5.416$, $p = 0.027$]. Neither of SMR, AS, EPOC, or T_R were related to any other component of the fast-start escape response (GLM, $p > 0.05$ in all cases). Acclimation temperature did not affect any of response latency, U_{max}, A_{max}, or turning rate (**Figure 3**; GLM, effect of temperature, $p > 0.05$ in all cases), and there were no significant interactions between temperature and any of SMR, AS, EPOC, or T_R.

Discussion

Our results show that fish responding to the stimulation with a short latency (i.e., fast reactors) tend to show long recovery times after exhaustive exercise and high AS. These results contrast with the "aerobic-scope driven" hypothesis that fast recovering individuals would be more willing to engage in burst-type locomotory behaviors. However, the results support the alternate "vigilance-driven" hypothesis that fish with the lowest latencies incur higher costs (and therefore longer recovery times after exercise) because they are most vigilant. Our results show that the shortest latencies were around 15 ms, in line with those recorded in Mauthner-mediated responses of other species (Eaton et al., 2001), while the longest latencies observed were around 50 ms and may be non-Mauthner responses (Kohashi and Oda, 2008). Vigilance is known to decrease the response time in prey (Krause and Godin, 1996), and in our experiments, the occurrence of a short latency is suggestive of high vigilance which may produce a lower threshold for a fast (Mauthner-cell mediated) response. A 30 ms difference between the shortest and the longest latencies is likely to provide an anti-predator advantage to fast reactors, since life or death may be determined by a matter of a few milliseconds during the initial phases of an escape response (Catania, 2009). This is especially true in structurally complex environments where escaping to the predator's first attack can allow the prey to hide and avoid a second attack. Juvenile golden gray mullet spent a large proportion of the year in lagoons, which are highly turbid and therefore avoiding the first attack may be fundamental for survival, since high turbidity can allow gray mullet to move out of the predator's visual field.

Highly vigilant individuals are likely to spend more energy because of higher activity in the brain (Roulin, 2001), one of the most metabolically active organs in the body (Roland, 1993; Rolfe and Brown, 1997). In vertebrates, vigilance is known to require increased oxygen consumption (Moss et al., 1998) and fish with no access to shelter show high oxygen consumption, at least partly because of increased vigilance (Millidine et al., 2006). Therefore, it is possible that highly vigilant individuals would have a relatively high metabolic rate after the being startled, and this would reflect in the longer recovery rate. Interestingly, neither SMR nor RMR were related to latency among individual fish. This suggests that increased vigilance may not

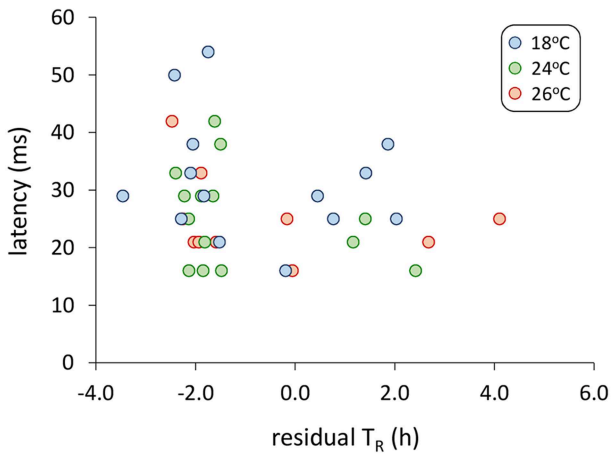

FIGURE 2 | Relationship between response latency during the fast-start escape response and time until 50% recovery after exhaustive exercise (T_R). For this visual representation, T_R is shown as residual values after correction for variation in body mass, though uncorrected values were used in the general linear model analysis presented in **Table 1**, with body mass as a covariate. GLM analysis revealed a significant effect of T_R on response latency [**Table 1**; $F_{(1,37)} = 7.06$, $p = 0.012$]. $n = 14$ fish per temperature.

TABLE 1 | General linear model results for the effects of body mass, standard metabolic rate (SMR), aerobic scope (AS), excess post-exercise oxygen consumption (EPOC), recovery time after exhaustive exercise (T_R), and temperature on latency during the fast start escape response in juvenile golden gray mullet.

Term	*df*	*F*	*p*	Estimate	Lower 95% CI	Upper 95% CI	*t*
Mass	1	2.96	0.095	1.145	−0.213	2.504	1.722
SMR	1	0.01	0.931	0.145	−3.226	3.516	0.088
AS	1	5.42	0.027	−2.841	−5.333	−0.348	−2.327
EPOC	1	3.39	0.076	0.005	−0.001	0.010	1.841
T_R	1	7.06	0.012	−3.120	−5.518	−0.723	−2.658
Temperature (26)	2	1.586	0.222	0			
(22)				−2.987	−11.119	5.144	−0.750
(18)				3.050	−5.484	11.583	0.730
Error	30						
Total	37						

Interactions between temperature and SMR, AS, EPOC, and T_R were included in the original model but removed when not significant.

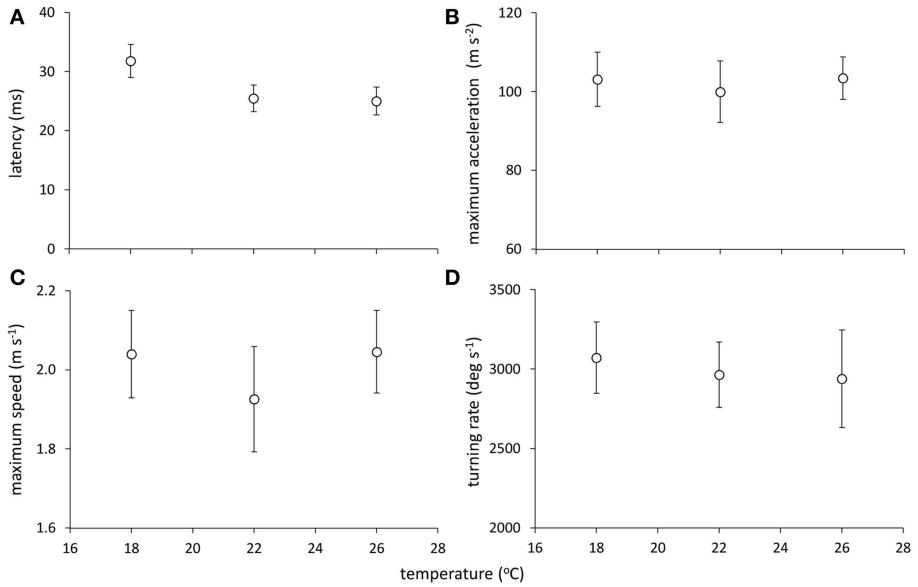

FIGURE 3 | Effect of temperature on components of the fast-start escape response in juvenile golden gray mullet: (A) response latency; (B) maximum acceleration; (C) maximum speed; and (D) turning rate. There was no significant effect of temperature on any response variable (see Results). Error bars = s.e.m; $n = 14$ fish per temperature.

incur increased costs while an animal is at rest, but that the costs may be context-dependent and especially pronounced in threatening situations (e.g., in the minutes or hours after an attack) when vigilance may be highest. It is also noteworthy that anaerobic capacity (as estimated by EPOC) had no relationship with response latency, and fast-start performance indices such as U_{max} and A_{max} were not related to recovery rate or AS. This is additional evidence that it is not energy expenditure during the escape response itself, nor the capacity to perform anaerobic exercise that is causing the prolonged recovery times among fish that respond sooner after an attack. Together these results suggest that a full assessment of the trade-offs related to high vigilance may need to include, in addition to the cost of lost feeding opportunities (Lima and Dill, 1990), the metabolic cost of vigilance (Millidine et al., 2006), which can result in a longer recovery phase after escape attempts.

The negative correlation between response latency and T_R suggests a functional trade-off between adaptations that enhance vigilance and responsiveness and those that permit rapid recovery of function following anaerobic exercise. A possible regulating mechanism is baseline levels of circulating glucocorticoids, which may be elevated in fast reactors. Cortisol, for example, is known to increase alterness and vigilance, and consequently escape behavior and cognitive performance in general (Johnson et al., 1992). Furthermore, high cortisol levels are usually associated with increased oxygen consumption (Davis and Schreck, 1997) which is in line with the long recovery times after exhaustive exercise observed in fast reactors.

Fast reacting individuals were also those with a relatively high AS. This result can also be interpreted in light of the increased cost of vigilance of such individuals. Having a high AS provides fast reactors with a wider scope for activity (Claireaux and Lefrancois, 2007), and therefore allows them to engage in metabolically costly physiological states, such as that related to high alertness. Their high AS means that an excess aerobic capacity beyond that needed to support high alertness is largely sustainable. Another possibility is that fish with a higher AS may also be more willing to engage in burst-type locomotion, given that recovery from anaerobic exercise is powered anaerobically. However, unlike some previous studies (Marras et al., 2010; Killen et al., 2014), we did not observe a statistically significant link between AS and T_R. Indeed, as previously discussed, it was the fish that responded slowest to the simulated attack that recovered fastest after exhaustive anaerobic exercise. Finally, it is conceivable that there was variability in the amount of exercise performed among individuals needed to achieve complete exhaustion, which was correlated with both the AS of individuals and their subsequent recovery time. Additional research could examine the effect of AS on recovery time after at a comparable level of anaerobic exercise intensity but below complete exhaustion.

The fact that the fastest responders are also the slowest to recover after anaerobic exercise suggests that, for these individuals, there is an additional cost associated with an early or premature flight response beyond lost foraging opportunities (Ydenberg and Dill, 1986). Anaerobic burst-type swimming causes a depletion of intramuscular glycogen, ATP, and phosphogen stores (Milligan, 1996; Kieffer, 2000). During recovery these fuels are replenished aerobically, a process which in fish is mainly fueled by lipid oxidation (Richards et al., 2002). Until recovery is complete, however, the ability to perform additional bursts of anaerobically-powered movement may be limited. Further, the rise in oxygen consumption that occurs during recovery from anaerobic exercise will occupy at least a portion of an

animal's AS, which could constrain other behaviors and physiological functions until recovery is complete. Therefore, although the behavioral decision to engage in costly burst-type activities may be vital for escaping predation, this type of locomotion can also carry appreciable costs.

Our results also show that golden gray mullet are resilient to changes in thermal acclimation within the range of temperatures examined in the current study. In general, the AS of fish peaks at some intermediate temperature and declines with acclimation to warmer or cooler temperatures (Fry, 1971; Claireaux and Lefrancois, 2007). The relatively wide breadth of temperatures over which golden gray mullet are able to maintain a high AS is likely an adaptation to life in lagoon environments, which can show large seasonal and even daily temperature fluctuations. The robustness of the fast-start response to acclimation temperature may also indicate that the vulnerability of juvenile golden gray mullet to predators is relatively constant a range of temperatures spanning 8°C. Furthermore, although not significant, our result show that latency tends to decrease with temperature, in agreement with previous work (Webb, 1978), and the idea that reaction time decreases with temperature because of slower nerve conduction speeds. Future work could examine how the escape response is affected by acute shifts in temperature.

In conclusion, our results show that individuals that take longer to recover after anaerobic exercise have a shorter escape response latency during a predator attack. After accounting for variation in other metabolic traits, fish with a higher AS are those that respond fastest to an attack. These findings indicate that individual metabolic traits may underlie variation in escape ability within prey species. Further, links between fast start ability and traits such as recovery ability may play a role in determining when individuals should choose to flee in a potentially dangerous situation. Additional work is needed to examine the ways in which the costs of engaging in anaerobic burst-type activity affects the economics of fleeing, beyond the traditional focus on lost foraging opportunities.

Author Contributions

SK, DR, SM, and PD conceived the study idea and designed the experiments. SK, DR, and SM conducted the experiments. SK, DR, SM, and PD contributed to the analysis of the data and the writing of the manuscript.

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Association between swimming performance, cardiorespiratory morphometry, and thermal tolerance in Atlantic salmon (*Salmo salar* L.)

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This experiment tested the hypothesis that swimming performance in Atlantic salmon (*Salmo salar*) parr is connected to cardiorespiratory performance and morphology, as well as maximum heart rate (f_{Hmax}) related measures of thermal tolerance. Moreover, it was hypothesized that the cardiorespiratory differences between poor and strong swimmers will be retained in a later life stage, i.e., 15 weeks post-smoltification and seawater transfer. This experiment screened a population of 3200 parr (11.2 ± 0.25 g) for their swimming performance, classifying them as poor and good swimmers based on their critical swimming speeds (4.4 ± 0.1 body length s^{-1} and > 6.8 ± 0.1 body length s^{-1}, respectively). Compared with poor performing parr, good swimmers had a significantly thicker compact myocardium (by 23.7%) and taller gill secondary lamellae (by 16.2%). In contrast, there was no significant difference in maximum oxygen consumption between the two groups as assessed using a "chase" protocol, and the relationship between heart rate specific measures of thermal tolerance and swim performance was variable. For example, three measures did not differ between the two groups, whereas the Arrhenius breakpoint temperature for f_{Hmax} and the highest f_{Hmax} value were lower and higher, respectively, in the poor swimmers. Importantly, the identified morphological and difference in the highest f_{Hmax} value at the parr stage persisted after 15 weeks of common garden rearing in seawater, and they were associated with an increase in relative ventricular mass and a small, but significant, improvement in growth rate. Therefore, it seems that an early assessment of swimming performance can effectively screen for morphological capacities related to oxygen supply and growth rate, but less so for heart rate related measures of thermal tolerance.

Keywords: screening fish, compact myocardium, swimming endurance, gill lamellae, optimum temperature, relative ventricle mass, maximum heart rate, oxygen consumption

INTRODUCTION

A formidable challenge for the salmon aquaculture industry is 10–20% mortality that occurs after smolts are introduced to sea cages. While much of the mortality is attributed to infectious and non-infectious diseases, fish in poor condition are often observed in sea-cages following smolt transfer and handling stress. These weaker individuals are assumed to be more susceptible for pathogens (Specker and Schreck, 1980; Iversen et al., 2005; Kristensen et al., 2012a). Central to the present study is the suggestion that an enriched rearing environment and training or conditioning early in parr development and prior to transfer to seawater could benefit the survival of farmed smolts (Anttila et al., 2011; Hyvärinen and Rodewald, 2013; Takle and Castro, 2013). This suggestion builds on the fact that swimming performance and cardiorespiratory physiology are plastic and appropriate exercise training programs have been shown to improve swimming capacity (Farrell et al., 1990; Davison, 1997; Gamperl and Farrell, 2004; Anttila et al., 2008), cardiac capacity (Farrell et al., 1991; Gallaugher et al., 2001; Castro et al., 2013a), and disease resistance (Castro et al., 2011, 2013a) of farmed fish.

Moreover, cardiorespiratory traits tend to show associations with each other. For example, swimming capacity correlates with cardiac capacity and oxygen transport capacity (Claireaux et al., 2005). Also, these traits are known to be important factors for lifetime fitness of wild salmon (Eliason et al., 2011, 2013), especially when extremely warm temperatures are concerned (Farrell, 2009; Eliason et al., 2011). Furthermore, cardiac morphology in farmed trout and salmon is distinct to wild conspecifics (Poppe et al., 2003; Gamperl and Farrell, 2004; Kristensen et al., 2012b). As a result, the Atlantic salmon aquaculture industry is searching for screening tools to improve the cardiac capacity of the smolts they chose for grow-out because better robustness could enhance survival and growth. Therefore, the purpose of the present investigation was to sort a population of 3200 Atlantic salmon parr

on their swimming performance and test the hypothesis that their swimming performance is connected to cardiorespiratory performance and morphology, as well as upper thermal tolerance. Furthermore, we examined whether or not any identified differences were retained into the critical post-smolt stage in seawater.

To test our hypothesis, we measured growth, indices of swimming performance, gill, and cardiac morphometry (e.g., ventricular mass, compact myocardium thickness, and gill lamellae height) and the response of maximum heart rate (f_{Hmax}) to acute warming before and after seawater transfer. While ventricular mass and gill lamellae height provide well-established indices of cardiorespiratory capacity, the comparison between rate transition temperatures for f_{Hmax} and swimming performance is novel. The rationale for selecting f_{Hmax} for the comparisons is that f_{Hmax} is a useful tool to rapidly characterize the upper temperature performance of small fishes. Heart rate (f_H) is the key cardiovascular response of fish to warming. Specifically, at temperatures beyond the optimal temperature (T_{opt}) for aerobic scope (difference between resting and maximum metabolic rate; Fry, 1947), f_H either reaches its maximum or begins to decrease (even becoming arrhythmic; Steinhausen et al., 2008; Sandblom et al., 2009; Eliason et al., 2011; Gamperl et al., 2011) and acts as a trigger for aerobic scope to fail (Farrell, 2009). Although not providing as much information as aerobic scope on the maximum capacity for energy availability for growth, swimming and immune defense (Pörtner and Farrell, 2008; Pörtner, 2010), associations are well-established between rate transition temperatures for f_{Hmax} and upper thermal tolerance indices for a variety of fish species, including a range of salmonids [e.g., coho salmon (*Oncorhynchus kisutch*), Casselman et al., 2012; rainbow trout (*Oncorhynchus mykiss*), Anttila et al., 2013a; sockeye salmon (*Oncorhynchus nerka*), Chen et al., 2013; pink salmon (*Oncorhynchus gorbuscha*), Clark et al., 2011].

MATERIALS AND METHODS

All procedures were approved by the National Animal Research Authority according to the "European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (EST 123).

EXPERIMENTAL FISH AND REARING CONDITIONS

Fish rearing and testing were conducted at the Nofima research station, Sunndalsøra, Norway. Fertilized Atlantic salmon (*Salmo salar* L., Bolaks strain) eggs were received from SalmoBreed AS (Bergen, Norway) at 386 day degrees and incubated at 7–8°C until hatching. In March 2013, emergent fry started feeding and temperature was progressively increased to 12°C according to industry standards. In May 2013, 3 weeks prior to swimming experiments, 3200 fish were selected within a narrow size range (to limit the variance in body mass to ±2 g) and moved to a single circular fiberglass tank (3 m diameter, 0.75 m water depth) supplied with the same water quality and temperature and reared under standard conditions (constant light, 85–100% oxygen saturation) with *ad libitum* feeding of a commercial feed (Skretting, Stavanger, Norway). The fish were sorted into poor and good swimmers according to swimming performance (as described below), and their maximum heart rate (f_{Hmax}) and metabolic rates were measured in June. Thereafter, the fish were kept in separate tanks for 4 weeks before being individually tagged (passive integrated transponder tag, Jojo Automasjon AS, Sola, Norway), measured for length and body mass before being reared in a single, common-garden tank during the smoltification period (short daylight period of 12:12 LD for 6 weeks followed by continuous light for 4 weeks). When the smolts were ready for seawater transfer by mid-September (according to standard smolt-tests), they were all re-measured for length and body mass and 100 good (average body mass 78 ± 7.4 g) and 100 poor (average body mass 76 ± 7.3 g) swimmers were selected for transport to the VESO Vikan Research Station (Vikan, Norway). At the research station fish were reared in seawater (25% salinity) for 15 weeks in a common garden aquarium (a single 1.5 m circular fiberglass tank) using standard culture conditions [flow through, 12°C, >80% oxygen saturation, 2% daily feeding of commercial feed (Skretting)]. In January 2014, the fish were again tested for length and body mass, and tested for maximum heart rate performance.

THE SCREEN FOR SWIMMING PERFORMANCE OF PARR

Swimming performance of 3200 parr was screened at 12°C in June 2013 after they had been starved for 24 h. Each screening test used 400 fish divided equally in two modified Brett-type swim tunnels supplied with the water of the same quality and temperature as the rearing tank. Therefore, the swimming performance was estimated for 16 batches of 200 fish. Each swim tunnel was composed of a 2 m long transparent PVC swim chamber with an inner diameter of 20 cm mounted in a leveled stainless steel frame. Each swim tunnel (approximate volume of 63 L) was supplied with water from an open fiberglass reservoir tank (7 m^3) via a 424 L closed polyethylene tank. The pump (VAKI Heathro Self Priming 6″ pump, VAKI Aquaculture Systems Ltd., Kópavogur, Iceland) supplying the water was controlled by a Cubix remote controller (HBC-radiomatic GmbH, Crailsheim, Germany). A laminar flow grid was also positioned in front of each swim chamber. The rear of each swim tube was connected to an external sling slip tee pipe, which drained the water back to open reservoir via a customized net to remove fish as they fatigued and no longer maintained station in the swim chamber. Water velocity was measured using a Micronics Portaflow 300 ultrasonic flow meter (Micronics Ltd., Buckinghamshire, UK). The swim protocol consisted of 30-min habituation to a low water velocity of 0.5 body lengths per second (*BL* s^{-1}). The fish were exposed to an incremental acceleration protocol that is thought to estimate critical swimming speed reasonably well (Farrell et al., 2003). The protocol consisted of a stepwise increase of 0.5 *BL* s^{-1} every minute up to 3 *BL* s^{-1}, which was maintained for 5 min before increasing the speed to 4 *BL* s^{-1} to position the fish in main water current. This speed was maintained until no more fish fatigued for a period of 10 min; fatigued fish were removed when they fell back into the net. Then the water speed was increased 0.5 *BL* s^{-1} every 5 min until only 30% of the fish remained swimming, which were termed good swimmers. This speed was noted and the 30% best swimmers were then given an enhanced and rapid acceleration (1 *BL* s^{-1} every 1 min) to remove them from the

swim chambers as they fatigued. The maximum velocity swum by the best swimmers was 1.2 m s^{-1} and the performance is simply reported as >6.78 *BL* s^{-1}. The first 30% of fish to fatigue were termed poor swimmers and the next 40% were termed moderate swimmers. After screening, all fish were fin clipped and good and poor swimmers were transferred to a new tank supplied with the same water as the holding tank for recovery. Parr recovered for at least 3 days after screening of swimming performance before the tests of f_{Hmax} and respirometry measurements were started. No mortality occurred during the 2-month rearing period following the swimming screen.

Swimming fish in bulk is the only practical way to screen swimming performance for a population of fish. While the intent was to measure swimming capacity, we cannot exclude the possibility that behavior and the willingness to swim were a component of this assessment (e.g., see Peake and Farrell, 2005, 2006; Farrell, 2007).

MAXIMUM HEART RATE MEASUREMENT IN PARR

The response of f_{Hmax} to acute warming was measured for 12 poor and 12 good swimmers (tested in a randomized order) as described by Casselman et al. (2012) with slight modifications. Briefly, each fish was first lightly anesthetized with 100 ppm MS-222 (buffered with sodium bicarbonate to pH 7.0) and weighed before placing it fully immersed in an experimental chamber that received temperature-controlled (11°C) aerated water from a Julabo circulating chiller/heater (F32 ME, Julabo GmbH, Seelbach, Germany). The circulating water, which contained 60 ppm buffered MS-222, was partially directed over the gills to maintain an anesthetized state throughout the whole experiment. An electrocardiogram (ECG) was recorded with a chromel-A measuring electrode positioned lightly on the skin just below the heart and a reference electrode positioned caudal to the heart. The ECG signal was amplified (1000×, Grass P55 amplifier, Astro-Med, Brossard, QC, Canada) and filtered (50 Hz line filter; low-pass: 30 Hz; high-pass: 0.3 kHz) before being stored in a PowerLab data acquisition system (PL3508, PowerLab 8/35, AD Instruments Ltd., Oxford, UK). Heart rate was allowed to stabilize for 30 min at 11°C before an intraperitoneal injection of atropine sulfate (2.4 mg kg^{-1} dissolved in 0.9% NaCl; Sigma-Aldrich, Oslo, Norway) that blocked vagal inhibition of the heartbeat. Preliminary tests showed that an isoproterenol injection did not increase f_H at any temperature as previously shown for Atlantic salmon (Anttila et al., 2014). Similarly, Casselman et al. (2012) found that isoproterenol increased f_H by only 0.3 beats min^{-1} in MS-222 anesthetized coho salmon. Water temperature was increased in 1°C increments for a cumulative warming rate of 10°C h^{-1}, beginning 15 min after the atropine injection. At each temperature increment both the water temperature and f_{Hmax} were stable. f_{Hmax} was recorded at each temperature increment by counting R–R intervals for final 15 heartbeats before another temperature increment. The incremental heating was terminated at the temperature when cardiac arrhythmias were first observed (QRS complex or P wave was missing) at which time the fish was quickly removed from chamber and euthanized by blow to the head prior to tissue sampling.

OXYGEN CONSUMPTION MEASUREMENTS OF PARR

Resting and maximum oxygen consumption rate (MO_2) measurements were performed on 10 poor and 10 good swimming fish (alternating tests). Each fish was transferred in the evening into either a 3.52 L Loligo swim tunnel respirometer (Loligo Systems ApS, Tjele, Denmark) or a 3.10 L respirometry chamber that were immersed in a water bath supplied with fresh water from the same source as the rearing tanks. Water in each chamber was continuously re-circulated and temperature in the chambers maintained at 12°C (± 0.5°C) for the duration of the experiments. After 1 h, while the fish adjusted to the chamber and recovered from handling, chambers were automatically cycled between being sealed for oxygen measurements (0.25 h) and being open for flushing (0.75 h). Thus, MO_2 was measured repeatedly overnight. Oxygen concentration (mg L^{-1}) was measured using an OXROB10 optical oxygen probe connected to a FireStingO2 oxygen meter (PyroScience GmbH, Aachen, Germany). Oxygen probes were calibrated daily. Blank runs without fish confirmed that background oxygen removal from the respirometer was negligible.

Maximum MO_2 measurements were performed during the following morning. Each fish was removed from its respirometer and transferred to 10 L chase tank. Fish were exhaustively exercised in the chase tank for 3 min and then air exposed for 1 min to ensure exhaustion. Fish were immediately transferred to a custom 0.27 L respirometry chamber and oxygen measurements began within 15 s. The chamber was immersed in a water bath and water in the chamber was circulated by a magnetic stir-bar in the bottom of the chamber that was separated from the fish by a plastic screen. Changes in MO_2 were measured using the same probe as above. Oxygen concentration in the chamber was allowed to decrease by a minimum of 10% and then chamber was manually flushed. Measurements were repeated for approximately 1 h or until MO_2 had decreased appreciably. The fish was then removed from the chamber and weighed before maximum MO_2 measurements were made on the next fish.

Resting and maximum MO_2 (mg O_2 kg^{-1} min^{-1}) were calculated from the decrease in oxygen concentration in the respirometry chambers. Resting MO_2 was calculated over a minimum period of approximately 500 s. Approximately 12 MO_2 measurements were made overnight and the resting MO_2 value was calculated as an average of the lowest three values for each fish (i.e., equivalent to a 25% quantile of the resting MO_2 records). Maximum MO_2 was calculated from a minimum period of approximately 50 s and was the highest value recorded, which typically occurred ∼2–5 min after the chase to exhaustion. Scope for MO_2 was calculated by subtracting resting MO_2 from maximum MO_2.

CARDIAC AND GILL MORPHOMETRICS OF PARR

The ventricle and the first gill arch from left side were removed after the f_{Hmax} tests ($N = 12$ per group). The ventricle was halved mid-sagittally. The gill and cardiac tissues were fixed in 4% formalin in phosphate buffered saline (PBS) for histological analyses. An additional 10 fish per group were sacrificed by blow to head 2 weeks after the swimming performance measurements to sample

the ventricle and compare the ventricle mass to the body mass (M_{RV}) of the fish.

Morphological assessments were made on all gill and ventricle samples ($N = 12$ per group). The formalin-fixed ventricles were dehydrated in alcohol and UltraClear (Mallinckrodt Baker Inc., Center Valley, PA, USA) series (70% EtOH 1 h, 94% EtOH 1 h, 100% EtOH 3 × 1 h, UltraClear 2 × 1 h) before embedding in paraffin wax. Serial sections (5 μm) were cut with microtome (301-268.001, Ernst Leitz GmbH, Wetzlar, Germany) and mounted on glass slides. The sections were de-waxed with UltraClear and rehydrated with an alcohol series and distilled water prior to staining with amylase–periodic acid–Schiff (PAS; Andersen, 1975). For PAS staining the sections were incubated for 1 h in 1% amylase at 37°C, rinsed with distilled water and oxidized with 1% periodic acid at room temperature for 20 min. After oxidation the sections were stained with Schiff's reagent for 20 min before rinsing and dehydrating them with alcohol and UltraClear series. The sections were examined in Leica DM RXA microscope (Leica Microsystems, Wetzlar, Germany). The thickness of the compact layer was measured from each ventricle at intervals (∼100 μm for parr and ∼300 μm for post-smolt) around the perimeter. The average compact thickness was divided by the cross-section area of the ventricle.

The formalin-fixed gill tissue were similarly processed prior to staining with Mayer's hematoxylin (RAL Diagnostics, Martillac, France) and using sagittal sections along the primary gill filament to provide cross-sections of the secondary lamellae. From these sections it was possible to measure the height of secondary lamellae in contact with water. Lamellar height was measured on at least six sections for each individual and the average was used to calculate the relative lamellar height by dividing it with fish mass.

TESTING AND MORPHOMETRICS OF POST-SMOLTS

After a 15-week grow-out period in seawater (i.e., 8 months after the sorting for swimming performance), f_{Hmax} of post-smolts from the poor and good swimming groups was measured as described above with slight modification. For post-smolts, heart rate was allowed to stabilize for 30 min at 13°C before the intraperitoneal injection of atropine sulfate to measure f_{Hmax} ($N = 7$ for both good and poor swimmers). After the f_{Hmax} measurements fish were euthanized and ventricles and gills were processed for histology as described above. The ventricular and body mass were also measured in these fish to calculate M_{RV}.

DATA AND STATISTICAL ANALYSES

Rate transition temperatures for the response of f_{Hmax} to acute warming were determined from each individual fish. The lowest rate transition temperature was an Arrhenius breakpoint temperature (T_{AB}), calculated according to Yeager and Ultsch (1989) by fitting two-segment linear regression lines to an Arrhenius plot [natural logarithm of f_{Hmax} and inverse of temperature (in Kelvin)] and calculating the intersection of the lines. The next highest rate transition temperature was the breakpoint temperature of Q_{10} (T_{QB}), which was established by first calculating the incremental Q_{10} of f_{Hmax} for each temperature step ($Q_{10} = [f_{Hmax\ n+1}/f_{Hmax\ n}]^{\wedge}\ [10/\{T_{n+1} - T_n\}]$), where $f_{Hmax\ n}$ was f_{Hmax} at a temperature n (T_n) and $f_{Hmax\ n+1}$ was f_{Hmax} at the next temperature (T_{n+1}). T_{QB} was the first breakpoint in a two-segment regression analysis of these Q_{10} data. The next highest rate transition temperature was the temperature (T_{max}) for the highest f_{Hmax} value (max f_{Hmax}). The highest rate transition temperature was arrhythmia temperature (T_{arr}), which was the lowest temperature to induced cardiac arrhythmia.

Statistical comparisons were made between poor and good swimmers, and between parr and post-smolts using Two-Way ANOVA followed by Holm–Sidak *post-hoc* test for M_{RV}, compact layer thickness, absolute and relative gill lamellae height, max f_{Hmax} and the rate transition temperatures (T_{AB}, T_{QB}, T_{arr}, and T_{max}). f_{Hmax} was compared for each temperature increment using Three-Way ANOVA with temperature, swimming group (good vs. poor) and age as factors. A Student's *t*-test was used to compare resting MO_2, maximum level of MO_2 and aerobic scope between poor and good swimmer parrs. One-Way ANOVA was also used to compare the weights and lengths of the poor and good swimmers at different time points. Data were $\log_{10}$ transformed whenever equal variance and normality assumptions were not met (compact thickness and total lamellae height). All the statistical analyses were performed with SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA). Statistical significance for comparisons of mean values was set at $\alpha = 0.05$. All values are reported as mean and S.E. unless otherwise stated.

RESULTS

SWIMMING PERFORMANCE, RESPIRATORY CAPACITY AND MORPHOLOGY OF PARR AND POST-SMOLTS

The screen for swimming performance segregated parr into poor (30%), moderate (40%), and good (30%) swimmers. Collectively, poor swimmers swam from 13 to 49 min to a final swimming speed of 4.39 ± 0.11 *BL* s^{-1} ($N = 960$). Moderate swimmers swam for almost twice as long, from 49 to 77 min, to a 55% higher final swimming speed of 6.78 ± 0.09 *BL* s^{-1} ($N = 1280$). Good swimmers swam for a minimum of 73 min and to an undetermined final swimming speed that was >6.78 *BL* s^{-1} ($N = 960$). Despite these differences in estimated swimming performance of parr, the body length, mass and condition factor for these performance groupings were indistinguishable (**Table 1**). Likewise, resting MO_2, maximum MO_2 and MO_2 scope were indistinguishable for the good and poor swimmers (**Table 1**).

Compared with poor swimmers, parr that were good swimmers had a thicker compact myocardium (by 23.7%) and significantly taller secondary gill lamellae (by 16.2%) (**Figure 1**). The relative height of the lamellae was 6.8 ± 0.4 and 6.2 ± 0.3 μm g^{-1} for good and poor swimmers, respectively, and did not differ statistically.

Only minor differences in thermal tolerance existed between poor and good swimmers. All parr, independent of swimming group, could be warmed to 23°C without the heart becoming arrhythmic. Yet, f_{Hmax} reached a significantly higher value at 21°C for poor swimmers compared with good swimmers, and this difference extended beyond 21°C for those fish hearts that did not become arrhythmic (**Figure 2A**, **Table 2**). However, the Arrhenius and Q_{10} break point analysis curves

(**Figures 2B,C**, respectively) revealed few and only minor differences in the rate transition temperatures, with only T_{AB} for f_{Hmax} being significantly different among the parr swimming groups (**Figure 2B**, **Table 2**).

The good swimmers of post-smolts had relatively larger ventricles (17.4%) and taller secondary lamellae (14.7%) as compared with poor swimmers (**Figure 1**). Furthermore, good swimmers had a 15.9% thicker compact myocardial layer (**Figure 1**) and the relative height of the gill lamellae was higher for good swimmers (0.65 ± 0.02 vs. $0.57 \pm 0.02\,\mu m\ g^{-1}$). In addition, they had developed a significantly larger ventricle (17.4%) (**Figure 1**). Also, similar to the parr stage, post-smolts of poor swimmers had significantly higher f_{Hmax} than good swimmers at temperatures beyond 21°C (**Figure 2A**). While all post-smolts ranked as poor swimmers maintained a rhythmic heartbeat up to 24°C, this was not the case for the good swimmers (**Figure 2A**). The rate transition temperatures for post-smolts did not, however, differ significantly between good and poor swimmers (**Table 2**).

When comparing the larger post-smolts with parr, f_{Hmax} at any given test temperature (**Figure 2A**) as well as max f_{Hmax} for post-smolts were significantly (50–52 bpm) lower than that for the parr. In addition, some rate transition temperatures differed significantly between parr and post-smolts. Specifically, T_{AB} was significantly higher and T_{max} significantly lower for post-smolts compared with parr (**Table 2**) and the Arrhenius and Q_{10} breakpoint temperatures were also more abrupt for post-smolts (**Figures 2B,C**).

While body mass and fork length did not differ significantly between poor and good swimmers at parr stage, a small, but significant difference emerged between poor and good swimmer at stages thereafter. The good swimmers were heavier (10.2–9.7%) as well as longer (2.9–3.6%) than poor swimmers as parr (4 weeks post swim-test [wps], $N = 960$) and as smolts (14 wps, $N = 960$). After the size grading prior to seawater transfer, the good swimmers tended to grow faster than the poor swimmers and they were 7.4% heavier and 3.4% longer than poor swimmers (29 wps, $N = 28$ per group) (**Figure 3**). No mortality was recorded during the experiment.

Table 1 | Swimming and metabolic capacities of good and poor salmon parr swimmers measured at 12°C.

	Poor swimmers	Good swimmers
Body mass (g)	12.3 ± 0.6	11.2 ± 0.4
Body length (*BL*, cm)	10.4 ± 0.1	10.1 ± 0.1
Condition factor	1.08 ± 0.01	1.09 ± 0.01
Swimming speed ($BL\ s^{-1}$)	4.39 ± 0.11	$> 6.78 \pm 0.09$*
Resting MO_2 ($mg\ kg^{-1}\ min^{-1}$)	1.5 ± 0.1	1.5 ± 0.1
Max MO_2 ($mg\ kg^{-1}\ min^{-1}$)	7.8 ± 0.2	7.9 ± 0.2
MO_2 scope ($mg\ kg^{-1}\ min^{-1}$)	6.2 ± 0.2	6.3 ± 0.2

Note: BL s^{-1}, body lengths per second; MO_2, oxygen consumption. $N = 3200$ for swimming performance and $N = 10$ per group for MO_2 measurements.
**Indicates significant difference between poor and good swimmers.*

DISCUSSION

The first aim of this study was to evaluate if Atlantic salmon parr could be sorted for swimming performance and was the swimming performance associated with cardiorespiratory performance and morphology as well as thermal tolerance. The present results revealed that it was possible to segregate parr of similar size into very distinct groups of good and poor swimmers with a simple screening test that used a 2 m long transparent swimming tunnel to test 200 fish at a time. Swimming performance of the good swimmers was minimally 55% better than the poor swimmers. While our intent was to measure swimming capacity, swim tests in general and in particular rapid screens such as this one do necessarily incorporate fish behaviors and willingness to swim. For example, it is possible that some fish positioned themselves in the water current to take advantage of other fish in terms of energy cost, just like human athletes do when racing competitively. Thus, while we think that the morphological and physiological associations with the swimming were most likely causative because we performed 16 independent tests to generate the fish groupings, we cannot be certain that a particular behavior (e.g., pro-active and reactive personalities) led to the morphological and physiological conditions that segregated out with the screen for swimming performance. Resolving this conundrum will require work well beyond the present study, which had as its primary aim to develop a useful test that could be used in aquaculture to screen for cardiorespiratory robustness. It is for

FIGURE 1 | Comparison of cardiac and gill morphometrics between swimming groups. (A) Relative ventricle mass (M_{RV}); which is ventricle mass in mg divided by mass of fish in grams, **(B)** relative thickness of the compact layer in ventricle which is calculated as thickness/ventricle cross-section area of ventricle, and **(C)** height of the secondary gill lamellae. *Indicates significant differences between good and poor swimming groups and the # indicates significant difference between parr and post-smolt in their respective swimming groups. $N = 12$ for parr and $N = 7$ for post-smolt per group.

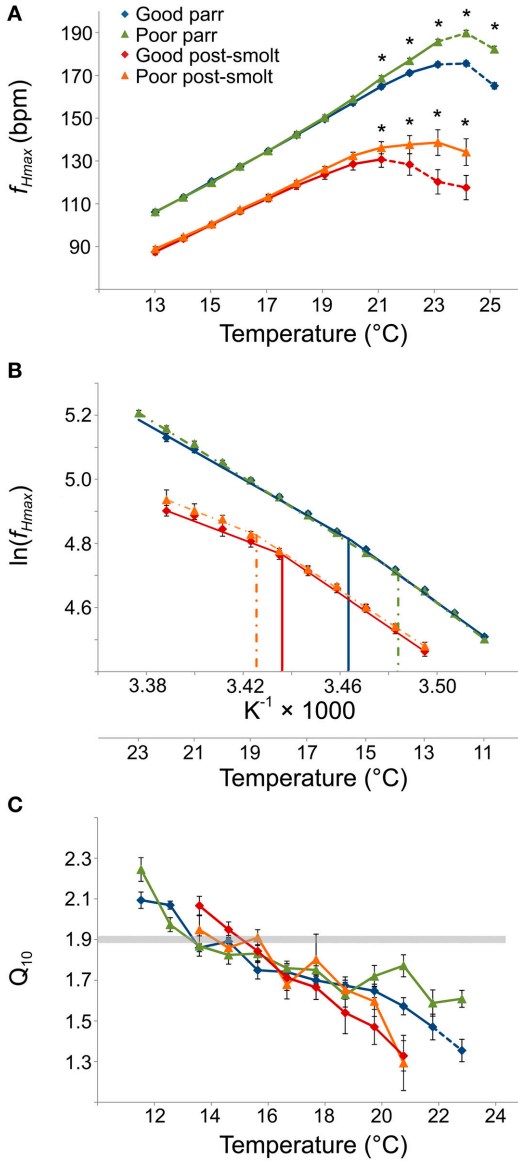

FIGURE 2 | (A) Maximum heart rates (f_{Hmax}) ± S.E. of the swim capacity groupings during acute warming. *indicates significant ($p < 0.05$) difference between good and poor swimmers. The parrs had significantly higher heart rate in each temperature point than post-smolt ($F = 48$, $p < 0.05$). The hatched line indicates the temperature where the first arrhythmias were observed. **(B)** Arrhenius plot of maximum heart rate (f_{Hmax}) of good and poor swimmers. The vertical lines indicate an Arrhenius break point temperature (T_{AB}) for the swim capacity groupings. **(C)** Average Q_{10} ± SE of maximum heart rate during acute warming. The gray bar ($Q_{10} = 1.9$) indicates the rate transition temperature where maximum heart rate starts an abrupt reduction in its exponential dependence with temperature, which is the Q_{10} break point temperature. In each figure, $N = 12$ per group for parr and $N = 7$ per group for post-smolt.

this reason that we use the term swimming performance rather than capacity to report the results of the screening test.

From an aquaculture perspective, the second aim of this study was to identify any features of cardiorespiratory robustness and evaluate whether or not any of the differences at parr stage persisted after a 15-week grow-out as post-smolts in seawater. The higher swimming performance of good swimmers was associated with the thicker cardiac compact layer and taller gill secondary lamellae, but with similar resting MO_2, maximum MO_2, and aerobic scope at 11°C. Importantly, these morphometric features were retained as post-smolts and additionally the good swimmers had developed a relatively larger ventricle. Furthermore, poor swimmers had a higher max f_{Hmax} without conferring any appreciable benefit to their upper thermal tolerance to acute warming; T_{QB} and T_{arr} were similar compared with good swimmers and T_{AB} showed only a minor difference. Of great importance to aquaculture was the discovery that better growth at the post-smolt stage was associated with good swimming performance. The potential application of these results to commercial aquaculture of Atlantic salmon is applying a simple screen for swimming performance at early age to improve robustness characteristics well beyond the smolt stage. Indeed, the morphological features and higher f_{Hmax} that distinguished good and poor swimming in 11 g parr persisted for 8 months and 15 weeks after seawater transfer. Moreover, good swimmers had improved growth rate. The disease-free aquarium environment used here did not, however, allow us to evaluate potential effects on fish survival. In fact, we observed no mortality. Nevertheless, a previous study with Atlantic salmon found that good swimmers had better survival than poor swimmers when challenged with a viral disease infection after smolt transfer (Castro et al., 2013b).

Beyond the novel insights of morphological differences in cardiorespiratory system as well as similarity of thermal tolerance between good and poor swimmers, the present findings support earlier information on the general cardiorespiratory associations when fish are sorted according to swimming performance. For example, good swimmers of rainbow trout had higher active metabolic rate and *in vivo* maximum cardiac output, but similar routine metabolic rate as poor swimmers at 16°C (Claireaux et al., 2005). In the present study, we do not see any difference in the metabolic rate measurements and have no explanation for this difference. Good swimmers in the earlier study also had a higher *in vivo* maximum cardiac output (the volume of blood heart is pumping per time unit) than poor swimmers, with heart rate and stroke volume both being numerically lower (but not reaching statistical significance) in the poor swimmers. This result is consistent with the present observation of a larger ventricular mass in good swimmers and similar f_{Hmax} for good and poor swimmers at cold temperatures. The previous study also showed that cardiorespiratory traits associated with better swimming performance can be retained in rainbow trout after a 9-month common garden grow-out in freshwater. Here, some but not all differences observed at the parr stage were retained in the post-smolt stage. Collectively, these results suggest that long-term experiments to adulthood are worthwhile to test how long such distinctions are retained. Also, we used an arbitrary 30% cutoff and do not know whether a greater proportion of the fish share these traits or to what degree the traits associated with poor swimmers were driven by a smaller proportion of the population. Thus, from an aquaculture perspective, a commercially viable screening for robustness should not cull too many parr and it would be useful to study if these traits are heritable, which

Table 2 | Comparison of rate transition temperatures (°C) derived from maximum heart rate (f_{Hmax}).

	Parr		Post-smolt	
	Good swimmers	**Poor swimmers**	**Good swimmers**	**Poor swimmers**
T_{AB}	$15.6 \pm 0.5^{a*}$	$13.9 \pm 0.4^{b*}$	17.9 ± 0.6^{A}	18.8 ± 0.6^{A}
T_{QB}	15.8 ± 0.4^{a}	$15.1 \pm 0.4^{a*}$	16.8 ± 0.4^{A}	16.3 ± 0.3^{A}
T_{arr}	26.1 ± 0.4^{a}	26.0 ± 0.4^{a}	25.3 ± 0.7^{A}	25.3 ± 0.7^{A}
T_{max}	$24.3 \pm 0.4^{a*}$	$24.4 \pm 0.2^{a*}$	21.1 ± 0.4^{A}	21.9 ± 0.4^{A}
max f_{Hmax}, bpm	$181 \pm 3^{a*}$	$192 \pm 2^{b*}$	131.4 ± 3.4^{A}	140.0 ± 3.4^{B}

*See text for the calculation of the T_{AB}, Arrhenius break point temperature; T_{QB}, Q_{10} break point temperature; T_{arr}, arrhythmia temperature; max f_{Hmax} absolute maximum heart rate; and T_{max}, the temperature where max f_{Hmax} was achieved. Different lowercase letters indicate significant differences between parr swimming groups and uppercase letters between post-smolt swimming groups. The * indicate significant difference between parr and post-smolts in their respective swim groups.*

N = 12 for parr and N = 7 for post-smolt per group.

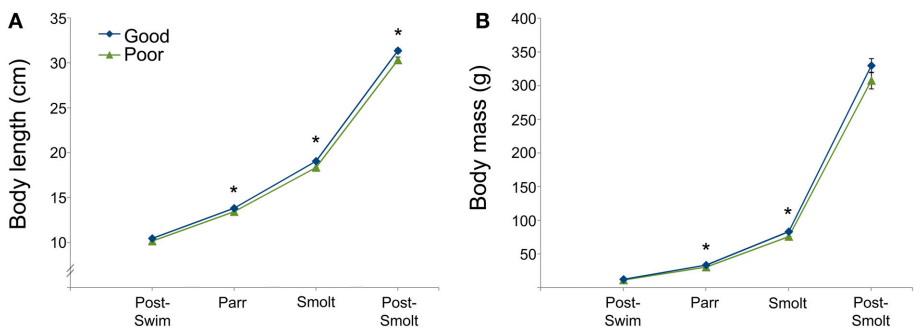

FIGURE 3 | The average (A) body length ± SE and (B) body mass ± SE of poor and good swimmers when tested at different time points after the swimming endurance experiment; directly after swimming (Post-Swim, *N* = 960 per group), at parr stage 4 weeks post-swimming (wps, *N* = 960 per group), at smolt stage 14 wps (*N* = 960 per group), at post-smolt stage (*N* = 28 per group). *Indicates significant difference between swimming groups.

would allow genetic selection as a strategy to improve cardiorespiratory robustness of the broodstock. Evidence already exists for heritability of thermal and swimming capacities (Garenc et al., 1998; Anttila et al., 2013b; Muñoz et al., 2014). To what degree heritable traits might be masked by the environmental plasticity of the cardiorespiratory system is equally fascinating.

Gills are regarded as a plastic organ and here taller gill secondary lamellae were associated with a greater swimming performance. Brauner et al. (2011) showed that exercise training in crucian carp (*Carassius carassius*) increased the lamellar surface area in contact with water, but without altering critical swimming speed, whereas goldfish developed a larger lamellar surface area and a higher swimming capacity when exposed to hypoxia (Fu et al., 2011). Of course, surgically removing gill lamellae has been shown to reduce critical swimming speed in rainbow trout (Duthie and Hughes, 1987). Thus, there are clear evidences for plastic responses of the gill with variable consequences to swimming performance. However, the present study is the first to relate it to a screen test for swimming performance.

The fish heart is similarly considered plastic within a species with respect to both its form and function (Gamperl and Farrell, 2004). For example, rainbow trout ranked as good swimmers had a more elongated ventricle and this translated to a greater cardiac output (Claireaux et al., 2005). By comparison, wild cohorts of Atlantic salmon have a more pyramidal ventricle compared with farmed fish (Poppe et al., 2003). The present work extends this idea by showing that good swimmers had a significantly larger relative ventricular mass at the post-smolt stage. This difference in ventricular mass could be largely a result of the greater proportion of compact myocardium, as seen in both the parr and post-smolt stage, which could supply the heart with a larger coronary circulation and assist contractile power. The finding that good swimmers have thicker compact myocardium is not surprising given the positive correlation found between ventricular volume and compact thickness in both rainbow trout and Atlantic salmon (Poupa et al., 1974) and that compact thickness is related to a higher athletic capacity of sockeye salmon (Eliason et al., 2011). Farmed gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), and Senegalese sole (*Solea senegalensis*) all had a thinner compact myocardium compared with their wild conspecifics (Pombo et al., 2012), suggesting a selective advantage for compact myocardium in the wild.

Another novel discovery was that the grouping according to swimming performance was not associated with any major difference in the acute upper thermal tolerance of parr or post-smolt, as indicated by f_{Hmax}. This finding has an important implication for aquaculture in that selecting fish with a high swimming performance could benefit cardiorespiratory robustness without

compromising upper thermal tolerance. The higher f_{Hmax} values at a given temperature of parr as compared to post-smolt were expected given the size and developmental difference (post-smolts were ∼26 times heavier). An inverse relationship between f_H and body mass is common among vertebrates (Lucas, 1994; Lillywhite et al., 1999) as is the reduction of the heart rate during development (Barrionuevo and Burggren, 1999). The f_{Hmax} measured after atropine injection in the present study show good agreement with the literature. Previously, Wood et al. (1979) observed a f_{Hmax} of ∼110 bpm at 20°C in 94–550 g rainbow trout, which is comparable to current value of 130 bpm of 300 g post-smolts. The f_{Hmax} of 157 bpm at 20°C for 11 g parr is again comparable to f_{Hmax} (∼140 bpm) of 10 g coho salmon (Casselman et al., 2012).

For juvenile salmonids, T_{AB} is a reasonable index for T_{opt} for aerobic scope while T_{arr} is a good index of upper thermal tolerance (Casselman et al., 2012; Anttila et al., 2013a). Here T_{AB} (∼ 15°C) of 11 g parr was comparable to the optimum growth temperature of Atlantic salmon parr (15.9°C; Elliott and Hurley, 1997) and T_{arr} (26°C) was below their incipient lethal temperature of 29°C (Beitinger et al., 2000). The slightly higher T_{AB} in post-smolts than in parr contrasts with juvenile turbot (*Scophthalmus maximus*) where the optimum temperature for growth rate decreases in larger fish (Imsland et al., 1996). The thermal tolerance window can be plastic and is expected to change with life stage (Pörtner and Farrell, 2008) and with thermal acclimation (Ferreira et al., 2014), which might help to explain the differences between parr and post-smolts here. For aquaculture purposes, the higher T_{AB} of good swimmers than poor swimmers at parr stage might also have a practical application as the good swimmers might be able to handle higher rearing temperatures than poor swimmers, an aspect worth further investigation.

An interesting discovery in the present study was the lower f_{Hmax} in the good swimmers at supra-optimal temperatures (> 21°C). This result needs to be considered in the context that cardiac output in fish can be increased through cardiac stroke volume as well as f_H (Farrell, 1991). Indeed, a larger cardiac stroke volume was observed earlier for good swimmers when maximum cardiac performance was tested *in situ* for farmed rainbow trout heart (Claireaux et al., 2005). Also, Franklin and Davie (1992) showed that rainbow trout with a larger ventricle have a larger stroke volume. Therefore, the elevated f_{Hmax} in poor swimmers may be directly related to their smaller ventricular mass (and perhaps a lower cardiac stroke volume) and a thinner compact myocardium compared with good swimmers.

In conclusion, important physiological and morphological differences between poor and good swimmers persisted for at least 8 months of rearing, suggesting that early screening for cardiorespiratory fitness traits in Atlantic salmon parr may represent a promising approach for salmon production.

AUTHOR CONTRIBUTIONS

Sven Martin Jørgensen, Gerrit Timmerhaus, and Harald Takle performed the sorting of the fish for swimming performance. Katja Anttila and Matthew T. Casselman performed the heart rate and MO_2 measurements of parrs and Sven Martin Jørgensen and Harald Takle performed heart rate measurements of post-smolts. Sven Martin Jørgensen, Gerrit Timmerhaus, and Harald Takle did the sampling of the fish at different time points for weight and length calculations. Katja Anttila did the morphological assessments. Katja Anttila analyzed the data of all the measurements. Harald Takle and Anthony P. Farrell initiated and provided the overall direction of the study and participated in writing the manuscript with considerable input from Katja Anttila and Sven Martin Jørgensen. All the authors have revised work critically for important intellectual content, have approved the version to be published and agreed to be accountable for all aspects of the work.

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Forced sustained swimming exercise at optimal speed enhances growth of juvenile yellowtail kingfish (*Seriola lalandi*)

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Swimming exercise at optimal speed may optimize growth performance of yellowtail kingfish in a recirculating aquaculture system. Therefore, optimal swimming speeds (U_{opt} in m s^{-1} or body lengths s^{-1}, BL s^{-1}) were assessed and then applied to determine the effects of long-term forced and sustained swimming at U_{opt} on growth performance of juvenile yellowtail kingfish. U_{opt} was quantified in Blazka-type swim-tunnels for 145, 206, and 311 mm juveniles resulting in values of: (1) 0.70 m s^{-1} or 4.83 BL s^{-1}, (2) 0.82 m s^{-1} or 3.25 BL s^{-1}, and (3) 0.85 m s^{-1} or 2.73 BL s^{-1}. Combined with literature data from larger fish, a relation of U_{opt} (BL s^{-1}) = 234.07(BL)$^{-0.779}$ (R^2 = 0.9909) was established for this species. Yellowtail kingfish, either forced to perform sustained swimming exercise at an optimal speed of 2.46 BL s^{-1} ("swimmers") or allowed to perform spontaneous activity at low water flow ("resters") in a newly designed 3600 L oval flume (with flow created by an impeller driven by an electric motor), were then compared. At the start of the experiment, ten fish were sampled representing the initial condition. After 18 days, swimmers (n = 23) showed a 92% greater increase in BL and 46% greater increase in BW as compared to resters (n = 23). As both groups were fed equal rations, feed conversion ratio (FCR) for swimmers was 1.21 vs. 1.74 for resters. Doppler ultrasound imaging showed a statistically significant higher blood flow (31%) in the ventral aorta of swimmers vs. resters (44 ± 3 vs. 34 ± 3 mL min^{-1}, respectively, under anesthesia). Thus, growth performance can be rapidly improved by optimal swimming, without larger feed investments.

Keywords: swimming exercise, growth, optimal swimming speed, feed conversion ratio, Doppler ultrasound imaging, aquaculture

INTRODUCTION

Aquaculture is facing an increasing demand for sustainably produced fish. There is a great need to optimize the conditions for fish growth, but without compromising health and welfare. Currently, significant numbers of commercially produced species of fish suffer from impaired well-being and high mortality (e.g., Castro et al., 2011, 2013). This may, at least partly, be explained by the fact that fishes cannot display their normal swimming behavior due to high densities or low water flow. A promising natural, non-invasive and economical tool to enhance growth may be the induction of swimming exercise at optimal swimming speeds (Palstra and Planas, 2011). Evidence suggests that the health and welfare of swimming fish is improved, not compromised (Castro et al., 2013; reviewed by Huntingford and Kadri, 2013).

Sustained swimming exercise improves growth in several teleostean fishes (Jobling et al., 1993; Davison, 1997; Palstra and Planas, 2011; Davison and Herbert, 2013). Exercise enhanced growth performance in salmonids, such as brook trout *Salvelinus fontinalis* (Leon, 1986; East and Magnan, 1987), brown trout *Salmo trutta* (Davison and Goldspink, 1977; Bugeon et al., 2003), rainbow trout *Oncorhynchus mykiss* (Greer Walker and Emerson, 1978; Houlihan and Laurent, 1987), Arctic charr *Salvelinus alpinus* (Christiansen et al., 1989, 1992; Grünbaum et al., 2008), and Atlantic salmon *Salmo salar* (Totland et al., 1987; Jørgensen and Jobling, 1993; Castro et al., 2011). Growth stimulation by swimming exercise has also been reported for non-salmonid species like yellowtail kingfish *Seriola lalandi* (Brown et al., 2011); gilthead seabream *Sparus aurata* (Ibarz et al., 2011; Sánchez-Gurmaches et al., 2013), whiting *Merlangius merlangus* (Hammer, 1994), striped bass *Morone saxatilis* (Young and Cech, 1993, 1994), qingbo *Spinibarbus sinensis* (Li et al., 2013) and the Amazon species matrinxa *Brycon amazonicus* (Arbeláez-Rojas and Moraes, 2010) and pacu *Piaractus mesopotamicus* (da Silva Nunes et al., 2013); also, zebrafish *Danio rerio* grow better when forced to swim (Palstra et al., 2010). The skeletal muscle thereby undergoes morphometrical and biochemical changes in response to exercise (Johnston and Moon, 1980; Davison, 1997; Johnston, 1999; Bugeon et al., 2003; Martin and Johnston, 2005; Rasmussen et al., 2013). Exercise increases muscle transcriptional activity underlying these changes, in particular genes involved

in muscle growth and developmental processes (Magnoni et al., 2013; Palstra et al., 2013). Exercise further enhances cardiac muscle growth and increases maximum cardiac output and hematocrit levels (rainbow trout; Farrell et al., 1990, 1991), all well-known adaptations to meet increased oxygen demand of tissues. Long-term sustained exercise lowers basal plasma cortisol levels in salmonids (rainbow trout: Woodward and Smith, 1985; Postlethwaite and McDonald, 1995, and Atlantic salmon: Boesgaard et al., 1993; Herbert et al., 2011) and striped bass (Young and Cech, 1993) and therefore cortisol may be a key player in exerting the exercise effects through its pivotal role in the control of metabolism and energy allocation (Mommsen et al., 1999).

Exercise-enhanced growth is optimal at a particular swimming speed where a maximum of energy is diverted to the skeletal muscles and where a minimum is lost due to other processes. The swimming speed for optimal growth is most likely near optimal swimming speeds (U_{opt}) where the cost of transport (COT, energy spent on swimming over a certain distance) is lowest and the energetic efficiency highest (Palstra et al., 2010; reviewed by Davison, 1997; Palstra and Planas, 2011; Davison and Herbert, 2013). Importantly, U_{opt} reflects very well the swimming speed for optimal growth in a variety of salmonid species and *Seriola* sp (reviewed by Davison and Herbert, 2013). At speeds below optimum, energy expenditure may increasingly go to activities such as aggression (type II allostatic overload: McEwen and Wingfield, 2003) and, at speeds above optimal, swimming soon becomes unsustainable and stressful leading to oxygen debt and eventually causing fatigue (reviewed by Davison, 1997; type I allostatic overload: McEwen and Wingfield, 2003). At U_{opt}, fish use the maximum of their energy for swimming and promoting the development of an aerobic phenotype. It may well be that cortisol, as a key player, warrants an optimal physiological stress condition (eustress) at U_{opt} which explains that also other beneficial effects may occur at this speed.

The carangid yellowtail kingfish (*S. lalandi* Valenciennes, 1883) is distributed circumglobally in subtropical seas, usually inhabiting deep pelagic waters (Nakada, 2002). Yellowtail kingfish swimming has a gross aerobic cost of transport comparable to that of swimming salmon or tuna species (Clark and Seymour, 2006). Migration capacity of *S. lalandi* is also similar: individuals were shown to travel over 2000 km from Australia to New Zealand (Gillanders et al., 2001). As a prized sushi and sashimi fish, *S. lalandi* is farmed in net pens in the USA, Chile, South-Africa, Japan and Australia, but also has excellent potential as viable new fish species for on-land culture in a recirculating aquaculture system (RAS; Abbink et al., 2012; Orellana et al., 2014; Blanco Garcia et al., 2014). So far, one study has investigated the effect of exercise on *S. lalandi* growth, showing 10% growth rate gain for fish of marketable size (1600 g) when exercised at 0.75 body lengths (BL) s^{-1} (Brown et al., 2011). This exercise was found optimal for growth stimulation of these fish when compared with 0, 1.5, and 2.25 BL s^{-1} (at 21.1 ± 0.03°C; 13L:11D; in ambient seawater in 13 m^3 tanks). Clearly, the potential of implementing exercise to improve growth rate in *S. lalandi* is indicated.

The first objective of this study is to assess the swimming performance of juvenile yellowtail kingfish, specifically the changes in optimal swimming speed during juvenile development. We subjected three size classes of juveniles to swim performance tests in swim-tunnels equipped with respirometers. Secondly, we then applied the relation between size and U_{opt} to determine the effects of long-term forced and sustained swimming at U_{opt} on growth performance. For this purpose we subjected two groups to either a water flow forcing fish to swim at U_{opt}, or to a low water flow that allows spontaneous activity. A newly designed 3600 L oval-shaped recirculating swim-flume was used to induce straight line swimming. The physiological consequences of exercise on growth were assessed by quantification of differences in size and blood flow. Importantly, both groups of fish were fed equal rations to avoid that feed intake would act as confounder in explaining anticipated physiological differences. In the light of earlier results (Brown et al., 2011), we hypothesize that forced sustained, straight line swimming exercise at U_{opt} will enhance growth performance of yellowtail kingfish and increase blood flow.

MATERIALS AND METHODS

ETHICS

All experiments were performed in accordance with relevant guidelines and regulations. Protocols used complied with the current laws of the Netherlands and were approved by the Animal Experimental Committee (DEC) of the Wageningen UR in Lelystad (The Netherlands) under numbers 2012012 and 2013162.

SWIM PERFORMANCE TESTS AND RESPIROMETRY IN SWIM-TUNNELS

Experimental fish and conditions

Juvenile yellowtail kingfish ($n = 33$; $BL = 88$ mm, $BW = \sim 8$ g) were obtained from the farm Silt BV (IJmuiden, the Netherlands). They were transported from the farm to the IMARES facilities in Yerseke by truck within 3 h. Fish were housed under similar conditions as in the hatchery: in natural seawater that was mixed with tap-water to 25.1 ± 0.1‰, at 23.4 ± 0.1°C and under a light regime of 16L:8D before and during the experimental periods. Fish were hand-fed three times per day at 7% BW day^{-1} with commercial feed (Skretting, Boxmeer, The Netherlands). Water quality was monitored daily for O_2, pH, NH_4, NO_2, and NO_3. Oxygen levels were 7.54 ± 0.08 mg L^{-1}, pH was 8.07 ± 0.07, NH_4-N averaged 0.27 ± 0.05 mg L^{-1}, NO_2-N averaged 1.5 ± 0.2 mg L^{-1} and NO_3-N averaged 8.9 ± 0.9 mg L^{-1} over the 2.5 months experimental period. During three periods in time, subsamples of this batch of fish were used for swim performance tests (size groups 1–3; **Table 1**).

Swim performance tests and respirometry

For the swimming experiments, two 127 L Blazka-type swim tunnels were used (van den Thillart et al., 2004). In each swim tunnel, water from the housing tank was circulating. A bypass with a galvanic oxygen electrode in a 4-channel respirometry system (DAQ-PAC-G4; Loligo Systems Aps, Tjele, Denmark) allowed registration of oxygen consumption. During respirometry, water in the swim-tunnels was recirculated and total oxygen content

Table 1 | Size of the experimental fish used for swim performance tests and respirometry in swim-tunnels.

Group	*N*	*BW* (g)	*TL* (mm)	*FL* (mm)	*K*
1	12	34 ± 3	145 ± 4	128 ± 4	1.06 ± 0.02
2	12	206 ± 14	206 ± 7	222 ± 6	1.26 ± 0.02
3	9	392 ± 16	311 ± 5	273 ± 4	1.31 ± 0.06

BW, body weight; TL, total length; FL, fork length. Significant differences existed in BW, TL and FL from one group to the next ($P < 0.05$).

dropped due to the oxygen consumption of the swimming fish. A low rate of background (bacterial) respiration was always detected and subsequently subtracted from fish oxygen consumption. The percentual decline of oxygen content was used to directly calculate COT according to the formula:

$$COT = \frac{\triangle sat_{(t)} \cdot mg_{O_2}}{m \cdot \triangle d} \quad (1)$$

where, $\triangle sat_{(t)}$ is the % decline in oxygen saturation during the measurement interval, mg_{O_2} is the amount of oxygen in mg per % saturation under the given conditions, m is the body mass of the fish in kg and $\triangle d$ is the covered distance in m. By equaling the first derivative of the polynomial function that described the relation between COT and the swimming speed U to zero, the U_{opt} was calculated.

Before each swimming experiment, two fish were individually introduced in a swim-tunnel and were allowed to acclimatize for 1 h which did not appear to be stressful for the fish as they were spontaneously swimming around at low speeds. Then, oxygen consumption was measured while swimming at consecutive speeds of 0.20, 0.40, 0.60, 0.80, and 1.00 m s^{-1} for 1 h per speed. At each speed, the fish were first allowed to acclimatize for 15 min before the oxygen content in the tunnels was measured for 30 min or until oxygen levels had dropped below 70% saturation. After each period of oxygen measurements the system was flushed to restore oxygen content to saturation levels. The experiment was terminated when either the fish fatigued (stopped swimming, hit the back fence and could not be stimulated to swim again) or when the fish had swum at 1.00 m s^{-1} for 1 h. Oxygen data from fish that stopped swimming at speeds <0.60 m s^{-1} were not used. Also data of some smaller fish that were observed to benefit from reduced flow by continuously swimming very close to the walls of the swim-tunnels at the highest speeds were excluded for those speeds. The oxygen consumption data were plotted against the swimming speeds in m s^{-1}.

After swimming, each fish was anesthetized in clove oil (1:10 diluted in absolute ethanol and used as 2 mL in 10 L water). The anesthetized fish was measured for total length (TL) and fork length (FL).

SWIM TRAINING TEST IN A SWIM-FLUME

The swim-flume, flow induction and turbulence

The experiment was conducted in a 3600 L oval-shaped Brett-type swim-flume (3.0 × 2.0 × 1.0 m; **Figure 1**; Brett, 1964) placed in a climatized room. The whole water volume of the recirculating system was pumped in 1 h over a filter system consisting of a Hydrotech drum filter (model HDF 501-1P, Hydrotech AB, Vellinge, Sweden), a trickle filter (Fleuren & Nooijen BV, Nederweert, the Netherlands), a 200 L biological moving bed biofilm reactor (MBBR) and a protein skimmer (Sander Aquarientechnik, Uetze-Eltze, Germany), all connected to a 400 L sump. Additionally, water from the 400 L sump was continuously pumped over a UV-filter (Proclear UV30 Advantage, Tropical Marine Centre Ltd. Hertfordshire, UK) and a heat exchanger (Maxicool XGL18HDA, Maxicool BV, Wessem, the Netherlands, modified by Climate4 u.nl, Valkenswaard, the Netherlands) to maintain water quality and water temperature, respectively. In one of the straight ends of the flume, two mesh fences (green polyester coated steel, 11 mm mesh size) were used to construct a compartment of 200 × 70 cm (**Figure 1C**). This compartment was divided by a PVC sheet (10 mm thick), thereby creating two 525 L sub-compartments, each measuring 200 cm ("x") × 35 cm ("y") × 70 cm ("z") (length × width × depth). The resulting inner sub-compartment, where water flow was nil, was used to house the resting fish. The outer compartment, where water flow was maximal, was used to house the swimming fish. Ample water circulation to maintain water quality in the inner compartment was ensured by installing a pump (Aqua Ocean Runner OR 6500, Aqua Medic, Loveland, CO, USA) in the curve that flushed water through the compartment at a rate of 6500 l h^{-1}.

Water flow was generated at the start of the straight end opposite the two compartments by an impeller connected to an electric motor (KLEEdrive MS2 132M-4 B3 (7.5 kW), Brd. Klee A/S, Albertslund, Denmark; **Figure 1**). The motor was powered by an industrial inverter (IP66, model no. BFI-E2-34-0180-3F4#, Beijer Electronics, Malmö, Sweden) with an adjustable alternating current (AC) output frequency between 0 and 50 Hz. This set-up to generate water flow was designed and first tested by Loligo Systems ApS (Tjele, Denmark). Additionally, a Speck pump (Badu 90/13, 0.55 kW with a capacity of 13 m^3 h^{-1}; Speck Pumps, Jacksonville, USA) continuously generated water circulation throughout the flume to safeguard water quality when the impeller was stationary.

Prior to the start of the experiment, water velocity in the swim compartment was measured using a downward-faced Vectrino acoustic Doppler velocimeter (ADV; Nortek AS, Rud, Norway) with its focal point at the center of the water column, 25 cm from the upstream fence. When the impeller was stationary, flow in the swimming compartment was measured. Inverter frequencies were subsequently increased with 0.5 Hz increments from 2.5 to 8.5 Hz, and after each increment, water flow was left to stabilize for 5 min, after which water velocities were measured in three dimensions (velocities u, v, and w in directions x, y, and z, respectively, as described above) for 10 s with a sampling rate of 10 Hz.

To quantify turbulence throughout the compartment, the inverter was set to 7.0 Hz, corresponding to a mean water velocity in the swimming compartment of 79 cm s^{-1}, after which water velocities were measured within the swimming compartment at three horizontal transects (25, 100, and 175 cm), three vertical transects (8, 17.5, and 27 cm) at three depths (15, 37, and 55 cm)

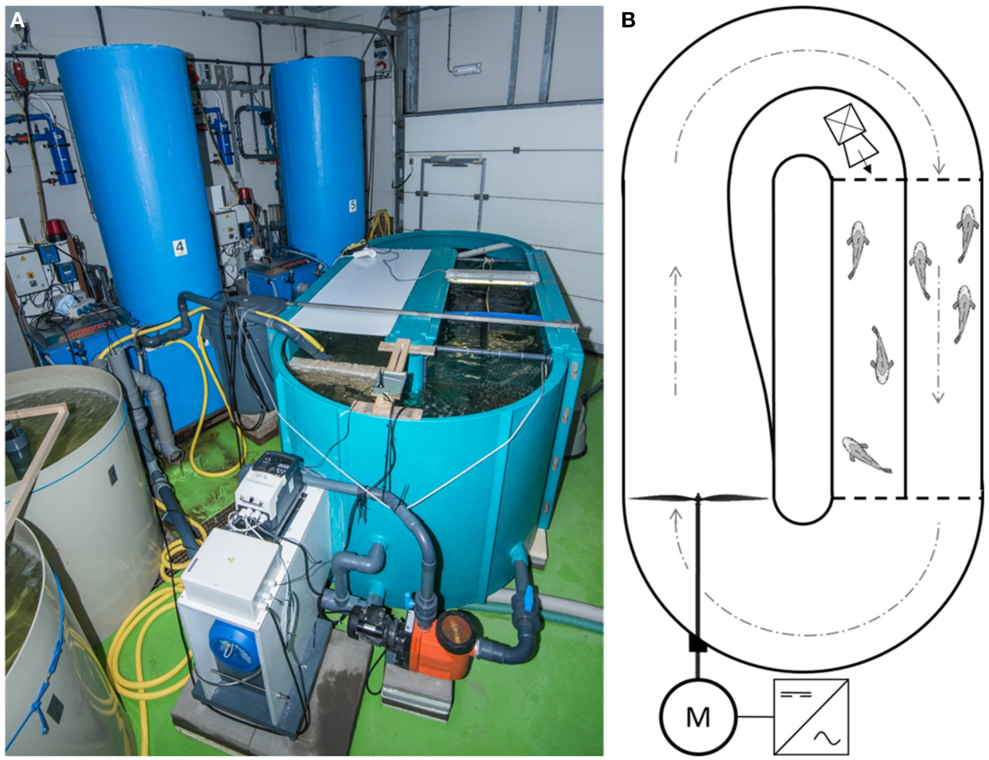

FIGURE 1 | The constructed swim-flume. (A) side-view; and **(B)** schematic overview. An industrial inverter provided an alternating current with adjustable frequency (Hz) to an electric motor, which in turn powered the impeller. Water was led through the swimming compartment, forcing fish ($n = 23$) to swim at the optimal swimming speed. Fish ($n = 23$) in the rest-compartment were not subjected to water flow and displayed spontaneous activity. To maintain high water quality in the rest-compartment, low water flow was generated by a small pump, which was placed in the curve outside the compartment. See text for detailed description.

which resulted in a total of 27 measurements. Using velocities u, v, and w, the dimensionless turbulence intensity (TI) was calculated according to Liao and Cotel (2013) at each of the 27 locations:

$$TI = u' / \left(\overline{u}^2 + \overline{v}^2 + \overline{w}^2\right)^{1/2} \quad (2)$$

where u' is the standard deviation of velocity u and $\overline{u}$, $\overline{v}$ and $\overline{w}$ are the average velocities in direction x, y and z, respectively.

Experimental fish and conditions

Juvenile yellowtail kingfish ($n = 56$) from the farm Silt BV (IJmuiden, the Netherlands) were randomly assigned to the rest-compartment ("REST"; $n = 28$) or the swimming compartment ("SWIM"; $n = 28$) of the flume and were acclimatized for 2 days.

After acclimatization, fish were not fed for 24 h and then ten fish (five from each compartment) were randomly selected and sampled (as "START" group). Individual fish were anesthetized in clove oil and total length (TL in mm) and body weight (BW in g) were measured from which Fulton's condition factor K was calculated, after which fish were euthanized by decapitation. The heart was dissected and heart weight (HW) was determined.

One day after the START group was sampled, the swimming trial commenced and the swimmers ($n = 23$) were forced to swim at U_{opt} in a sustained manner while resters ($n = 23$) were allowed to perform spontaneous activity at low water flow. Fish were fed commercial feed (Efico Sigma 570 No 6.5, Biomar A/S, Brande, Denmark), three times per day on weekdays and twice per day during weekends. The water flow was stopped during feeding sessions. First, the resters were fed until apparent satiation and the amount of feed given was determined. Then, swimmers were pair-fed and received the same amount as the resters. After feeding, fish were left for 15 min and then the water velocity was gradually increased to U_{opt}. Fish were fed 2.65 ± 0.13 % BW d^{-1} and food conversion ratio (FCR) for both treatments was calculated as the ratio of biomass gain (wet weight in g) to total food intake (g).

Fish were checked for swimming behavior at least twice per day. Experimental fish did not show any visible signs of stress or fatigue, nor did infections, disease, or mortality occur. During the experiment, salinity was maintained at 26.2 ± 0.2‰ by mixing natural seawater (from the estuary Oosterschelde) with tap water. Mean water temperature was maintained at 23.6 ± 0.1°C. Oxygen gas was supplied through a ceramic diffuser downstream of the two compartments. Hundred percentage system volume was replaced daily to ensure high water quality. Water quality was monitored daily for O_2, pH, NH_4 and NO_2, while NO_3 was checked seven times throughout the 18-day experimental period. Oxygen levels were 7.67 ± 0.07 mg L^{-1}, pH was 7.42 ± 0.029, NH_4-N averaged 0.8 ± 0.2 mg L^{-1} (min 0 mg L^{-1} and max 2.3 mg L^{-1}), NO_2-N averaged 1.4 ± 0.2 mg L^{-1} (min 0.2 mg L^{-1} and max 3.1 mg L^{-1}) and NO_3-N averaged 14.8 ± 3.5 mg L^{-1}

(min 2.01 mg L^{-1}, measured after replacing one system volume, max 19.5 mg L^{-1}, measured after 16 h without any water replacement) over 21 days. Nitrogenous waste-products were within safe limits for yellowtail aquaculture (Pierce et al., 1993; Colt, 2006).

After the 18-day experimental period, fish were not fed for 24 h, after which ten fish per treatment were collected and sampled as described for the START group. Of the remaining fish ($n = 13$ per treatment), blood flow was determined by Doppler ultrasound imaging, using an Esaote MyLabFive Vet ultrasonography unit (Esaote Europe BV, Maastricht, the Netherlands) with a 18 MHz LA 435 ultrasound transducer (Easote). Blood flow was visualized using the brightness (B) mode, Colour Flow Mapping (CFM) and Pulse Wave (PW) with the following settings: Velocity 89%; Angle (θ) + 60°; Depth 6 cm, $Gain_B$ 76%, $Gain_{CFM}$ 70%, $Gain_{PW}$ 52%; $Frequency_B$ 18 MHz, $Frequency_{CFM}$, 8 MHz, and $Frequency_{PW}$ 8 MHz. Anesthetized fish were positioned on a table with the right lateral side facing upwards. The ultrasound transducer was covered with Aquasonic ultrasound transmission gel (Parker Laboratories Inc., Fairfield, NJ, USA) and held motionless against the ventral side of the fish near the head, in a longitudinal direction, allowing visualization of blood flow in the ventral artery, just downstream of the *bulbus arteriosus* (Figure S1). The scanning procedure was completed within 2 min. Blood flow was determined using the "El-Flow" function of the ADV. Using this function, the user manually traces the contour of the blood velocity graph and the width of the blood vessel is indicated. The ADV subsequently calculates the time average velocity as well as the cross-sectional area of the vessel, assuming a circular shape. Using these two parameters, the ADV calculates blood flow in mL min^{-1}. After ultrasonography, fish were measured for TL and BW, after which fish were euthanized by decapitation.

Size measurements were used to calculate weight-specific growth rates for both swimmers and resters:

$$SGR_w = \left(\ln\left(W_f\right) - \ln\left(W_i\right)\right) \times \frac{100}{t} \quad (3)$$

where W_f is the final average weight of either resters or swimmers (g), W_i is the initial average weight of the START group (g) and t is time between measurements (d).

STATISTICS

BW, TL, and FL data in part I (Swim performance tests and respirometry in swim-tunnels) were normally distributed and tested for differences occurring between the three size classes (ANOVA, $P < 0.05$). K data were tested with Kruskal–Wallis tests.

All data in part II (Swim training test in a swim-flume) showed normal distribution (Shapiro–Wilk tests). To test for background effects over time, resters were compared to the START group. To test for treatment effects, swimmers were compared to resters. TL and BW were compared using student's *t*-tests with one-tailed probabilities, while K was compared using Mann–Whitney *U*-tests with two-tailed probabilities. Analysis of covariance (ANCOVA) with BW as the cofactor was performed on log transformed unpaired observations in search for group effects in the parameters HW and BF. Parameters were compared between resters and the START group and between swimmers and resters. In case there was no significant effect of the cofactor BW, ANOVA was used to determine whether there was a group effect.

Differences with $P < 0.05$ were considered significant. All data are presented as mean ± standard error (SE).

RESULTS

SWIMMING PERFORMANCE TESTS AND RESPIROMETRY IN SWIM-TUNNELS

Size

Size parameters (BW, TL, FL, and K) significantly increased from one experimental group to the next ($P < 0.05$; **Table 1**).

Swimming behavior

Seven fish of the smallest size group 1 at speeds of 0.80 m s^{-1} benefited from reduced drag by continuously swimming close to the walls of the swimming tunnels. One fish of group 1, two of group 2 and one of group 3 could not swim and appeared stressed in the swim-tunnels. Three fish of group 1 (critical swimming speed $U_{crit} = 0.30 \pm 0.06$ m s^{-1} or 2.16 ± 0.47 BL s^{-1}; calculated like Brett, 1964), three fish of group 2 ($U_{crit} = 0.71 \pm 0.15$ m s^{-1} or 2.61 ± 0.53 BL s^{-1}) and one fish of group 3 ($U_{crit} = 0.60$ m s^{-1} or 1.88 BL s^{-1}) fatigued before swimming 1 h at 1 m s^{-1}. One fish of group 1 was able to swim 1 h at 1 m s^{-1} (equal to 6.21 BL s^{-1}) without swimming close to the walls, as well as seven of group 2 (represents 58% of the fish; equal to 4.12 ± 0.15 BL s^{-1}) and seven of group 3 (represents 78% of the fish; equal to 3.21 ± 0.06 BL s^{-1}).

Cost of transport (COT) and optimal swimming speeds (U_{opt})

The calculated cost of transport (COT) values were plotted against the swimming velocities as polynomial *U*-shaped curves for each of the subsequent groups (**Figures 2A–C**). Only in case of the smallest size group 1 the polynomial curve was actually the best fitting ($R^2 = 0.9935$). For groups 2 and 3, most of the fish were still swimming at 1 m s^{-1} so that the minimal COT value determining U_{opt} may not have been reached. Polynomial curves (for groups 2 and 3, respectively, R^2-values of 0.9624 and 0.9669) were plotted to calculate U_{opt}-values for both groups. The average COT was higher in group 1 as compared to the fish of groups 2 and 3 (**Figure 2**). The polynomial for group 1 followed the equation $y = 9.9503x^2 - 13.924x + 5.9924$; for group 2 the equation was $y = 1.9141x^2 - 3.1348x + 1.5051$; and for group 3 the equation was $y = 2.6024x^2 - 4.3019x + 2.044$ (**Table 2**). The absolute U_{opt} (in m s^{-1}) increased for each of the subsequent size groups from 0.70 m s^{-1} for group 1; to 0.82 m s^{-1} for group 2; to 0.85 m s^{-1} for group 3 (**Table 2**). The relative U_{opt} (in BL s^{-1}) decreased: 4.83 BL s^{-1} for group 1, 3.25 BL s^{-1} for group 2, and 2.73 BL s^{-1} for group 3 (**Table 2**).

SWIM TRAINING TEST IN A SWIM-FLUME

Flow and turbulence

Once the impeller was stationary, flow in the swimming compartment was 8.06 ± 0.27 cm s^{-1} as a result of the circulation generated by the Speck pump. Horizontal water velocity (u) in the swimming compartment increased proportionally to the AC-frequency of the inverter ($R^2 = 0.9977$; Figure S2). Turbulence intensity (TI) in the swim compartment was subsequently calculated as the average over the 27 measurements. At a mean water

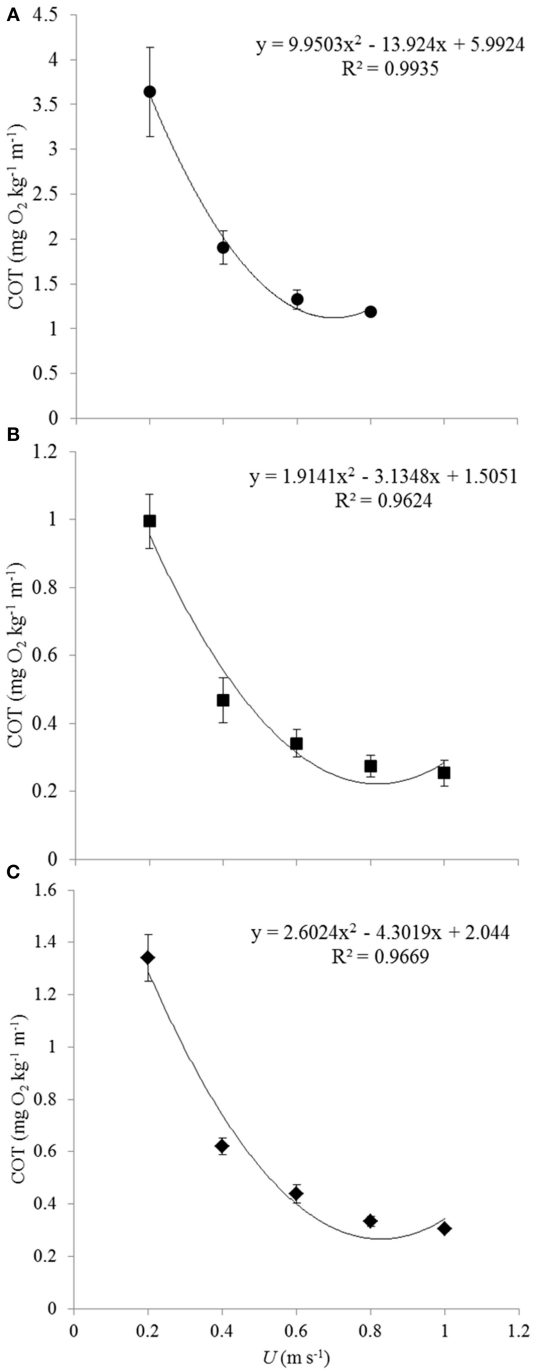

FIGURE 2 | Average COT values for each of the three size groups and polynomial plotting of trend-lines. Each of the graphs representing the subsequent size groups gives the polynomial relation between COT and U and the R^2-value. Averages and standard errors are based on data of decreasing numbers of fish for increasing speeds because of fish fatiguing and because of rejecting data of fish that were utilizing the lower flows near the wall of the swim-tunnel: **(A)** 0.20 m s^{-1}: $n = 7$; 0.40 m s^{-1}: $n = 7$; 0.60 m s^{-1}: $n = 6$; 0.80 m s^{-1}: $n = 2$, **(B)** 0.20 m s^{-1}: $n = 5$; 0.40 m s^{-1}: $n = 8$; 0.60 m s^{-1}: $n = 8$; 0.80 m s^{-1}: $n = 7$; 1.00 m s^{-1}: $n = 6$, **(C)** 0.20 m s^{-1}: $n = 7$; 0.40 m s^{-1}: $n = 7$; 0.60 m s^{-1}: $n = 7$; 0.80 m s^{-1}: $n = 6$; 1.00 m s^{-1}: $n = 5$. COT values of the smaller fish of group 1 were generally much higher than those of groups 2 and 3. Individual variation in COT decreased at increasing swimming speeds.

velocity of 79 cm s^{-1} in the swimming compartment, TI was 0.083 ± 0.0032.

Swimming behavior

Swimmers displayed rheotactic behavior, grouping together in a school near the upstream fence of the compartment. The flow created by the impeller forced these fish to swim sustainably. Fish in the resting compartment displayed spontaneous swimming activity. After 18 days of continuous swimming, swimmers had swam an equivalent distance of 1250 km.

Fish growth

Mean TL of the START group was 346 ± 6 mm and U_{opt} for this size class of fish was determined at 2.46 BL s^{-1} (water velocity of 0.85 m s^{-1}). After 18 days at U_{opt} speeds, swimmers had grown an average 39 mm to 385 ± 4 mm whilst resters only grew 21 mm to 367 ± 5 mm; a 92% greater increase in length ($P < 0.05$; **Figure 3**). Furthermore, swimmers (735 ± 23 g) increased 231 g in BW as compared to the START group (504 ± 27 g), which is a 46% greater increase than the resters (661 ± 32 g) ($P = 0.035$; **Figure 3**). SGR was 40% higher for swimmers (2.1% BW d^{-1}) as compared to resters (1.5% BW d^{-1}). Swimmers and resters had similar condition factors ($K = 1.28$ and 1.32, respectively) ($P > 0.05$; **Figure 3**). Feed conversion ratio was lower and thus more efficient at 1.21 compared to 1.77 in resters.

Heart and blood

HW (with BW as cofactor) showed any significant differences between groups (**Table 3**). Swimmers had a blood flow of 44 ± 3 mL min^{-1}, resters 34 ± 3 mL min^{-1} (**Table 3**). Swimmers thus showed a significantly higher (+31%) cardiac output in the ventral artery ($P = 0.026$; **Table 3**).

DISCUSSION

In this study we have established optimal swimming speeds for juvenile yellowtail kingfish allowing to apply this knowledge for an experimental swim-training trial of 18 days to investigate the effects of swimming exercise on growth performance and cardiac output. Exercise-enhanced growth was robust and not caused by increased feed intake. It may have been caused by increased feeding efficiency indicated by a lower FCR. Doppler ultrasound imaging showed a cardiac output which was significantly higher in exercised fish, no such data have been shown for fish before (to the best of our knowledge).

Plotting polynomial curves for each of the three size classes enabled us to calculate the swimming speeds at which COT was minimal and that correspond to the U_{opt} (Palstra et al., 2008). Calculating these speeds revealed increasing absolute U_{opt}-values in m s^{-1} with increasing body length which were decreasing when expressed relative to body length (**Table 2**). As most of the larger fish were still swimming at 1 m s^{-1}, polynomial curves predicted by the classical exponential relation between U and MO_2 (Jones and Randall, 1978), were not the best fitting curves for groups 2 and 3. Values were however well in line with the two U_{opt}-values known from literature for larger *S. lalandi*: 2.25 BL s^{-1} for yellowtails of 362 mm BL (Brown et al., 2011) and 1.7 BL s^{-1} for yellowtails of 569 mm BL (Clark and Seymour, 2006). By plotting the three U_{opt}-values for juveniles of this study with these

Table 2 | Optimal swimming speeds (U_{opt}) per group in m s^{-1} and BL s^{-1}.

Group	*y(x)*	*y′(x)*	U_{opt} (m s^{-1})	U_{opt} (BL s^{-1})
1	$y = 9.9503x^2 - 13.924x + 5.9924$	$19.9006x - 13.924 = 0$	0.70	4.83
2	$y = 1.9141x^2 - 3.1348x + 1.5051$	$3.8282x - 3.1348 = 0$	0.82	3.25
3	$y = 2.6024x^2 - 4.3019x + 2.044$	$5.2048x - 4.3019 = 0$	0.85	2.73

Polynomial equations are shown for COT vs. U and their first derivatives. U_{opt} is defined as the speed with lowest COT and calculated by equaling the first derivative of each of these equations to zero. U_{opt} is given in absolute terms in m s^{-1} and in relative terms as BL s^{-1}. The absolute U_{opt}-values increase while the relative U_{opt}-values decrease at increasing size.

two U_{opt}-values for larger fish, a relation can be established of U_{opt} (BL s^{-1}) = 234.07(BL)$^{-0.779}$ ($R^2 = 0.9909$; **Figure 4**). This formula describes the relation between body length and optimal swimming speed over a size range of 145–569 mm BL and therewith provides a tool to calculate U_{opt}-values for fish in this size range.

Eighteen days of sustained exercise training at optimal swimming speed enhanced growth of juvenile yellowtail kingfish substantially with a 92% gain in length and a 46% gain in body weight as compared to the increase seen in controls. Because resters and swimmers were housed in the same flume under the same conditions and given equal feed rations, increased SGR_w in swimmers is predicted to be a result of sustained swimming exercise alone. Our results support a growing body of work that shows growth-stimulating effects of sustained exercise in active metabolic fish, mainly salmonids and pelagic teleosts (reviewed by Davison and Herbert, 2013). Typically, the enhancement of growth performance (e.g., increase in body weight) by exercise is ∼40% in these species (Davison and Herbert, 2013). Brown et al. (2011) found exercise-enhanced growth performance in *S. lalandi* but only a moderate 10% increase in SGR_w. This result is contrary to the results of Yogata and Oku (2000) who found a 36–38% weight gain in exercised fingerling *S. quinqueradiata* which better fits the expectation. Brown et al. (2011) speculated on the difference in growth stimulation comparing their study with the study of Yogata and Oku (2000), e.g., a suboptimal temperature (21.1 vs. 22.0–24.6°C, respectively) and larger size of the fish (1600 vs. 4 g, respectively). The most appropriate explanation may actually be the lower applied flow: 0.75 BL s^{-1} vs. 1.0–2.25 BL s^{-1}, respectively, although body size and temperature may also affect the relationship between U_{opt} and growth in *Seriola* sp. According to the functional relation that we found, the U_{opt} for the fish used by Brown et al. (2011; 476 mm BL) would be 1.92 BL s^{-1} and thus was the reported speed for optimal growth, suboptimal in respirometric terms (although routine speed was not recorded, estimated to be slightly lower at ∼0.5–0.75 BL s^{-1}, and calculated to correspond to 1.9–2.4 BL s^{-1} when swimming in a straight line which would correspond to the U_{opt} that can be calculated with the functional relation that we found). We believe that our study shows exercise-enhanced growth for *Seriola* spp. of 46% because fish were forced to swim at their *optimal* swimming speed. This also agrees with the deduced highest weight gain in fingerling *S. quinqueradiata* as reported by Yogata and Oku (2000; ∼45% in **Figure 1**). From our functional relation we can however not determine what would be U_{opt} for fish <145 mm and thus we do not know which would be U_{opt} for the fingerlings used by Yogata and Oku (2000). The reason why Brown et al. (2011) did not find more pronounced growth stimulation at the higher applied swimming speeds of 1.5 BL s^{-1}, and perhaps also at 2.25 BL s^{-1}, remains however a mystery according to the hypothesis that growth stimulation would occur at U_{opt}. Our findings may support this hypothesis.

Sustained exercise has been demonstrated to improve food conversion rates (FCRs) in several active teleosts (Jobling et al., 1993; Davison, 1997; Magnoni et al., 2013). The present study revealed a 32% lower FCR for swimmers as compared to resters, supporting previous studies on *S. lalandi* (Brown et al., 2011) and *Seriola quinqueradiata* (Yogata and Oku, 2000). The mechanisms driving reduced FCRs in exercised fish are not yet fully scrutinized, but recent studies suggest that exercise increases nutrient uptake efficiency. For example, exercise increased carbohydrate turnover and promoted protein-uptake in white muscle in gilthead sea bream *S. aurata* (Felip et al., 2013) and in Atlantic salmon *Salmo salar*, higher energy efficiency and amino acid synthesis were detected in exercised smolts as compared to resting fish (Grisdale-Helland et al., 2013). Although our understanding of improved FCRs under exercise regimes is still limited, our results indicate that the implementation of sustained exercise in aquaculture has the potential to extensively improve growth performance and reduce feeding costs at the same time.

Heart weights did not increase as a treatment effect but what we did find was a significantly increased blood flow in the ventral artery in exercised fish, just after the *bulbus arteriosus*, reflecting a higher cardiac output. Although aerobic exercise has been shown to increase cardiac growth in some teleosts, effects are often small and variable (reviewed by Gamperl and Farrell, 2004). *S. lalandi* has previously been shown to increase cardiac output by exercise training, without increasing cardiac stroke volume but by increasing heart rate (Clark and Seymour, 2006), which supports a higher blood flow without the increase of HW as found in the current study. A similar observation was made in rainbow trout, where daily exercise cycles did not increase heart size (Farrell et al., 1990) but did increase heart capacity in exercised fish (Farrell et al., 1991).

Ultrasound imaging is extensively used for non-invasive gonadal observations and sex identification in teleost fishes (reviewed by Novelo and Tiersch, 2012). Currently, teleost blood flow is often determined by surgical insertion of Doppler flow probes, which requires deep anaesthetization of the animal, invasive surgical procedures with risks of infection, and leaves the fish with leads protruding from the body, which have to be connected to velocimeters (e.g., Thorarensen et al., 1993; Clark and

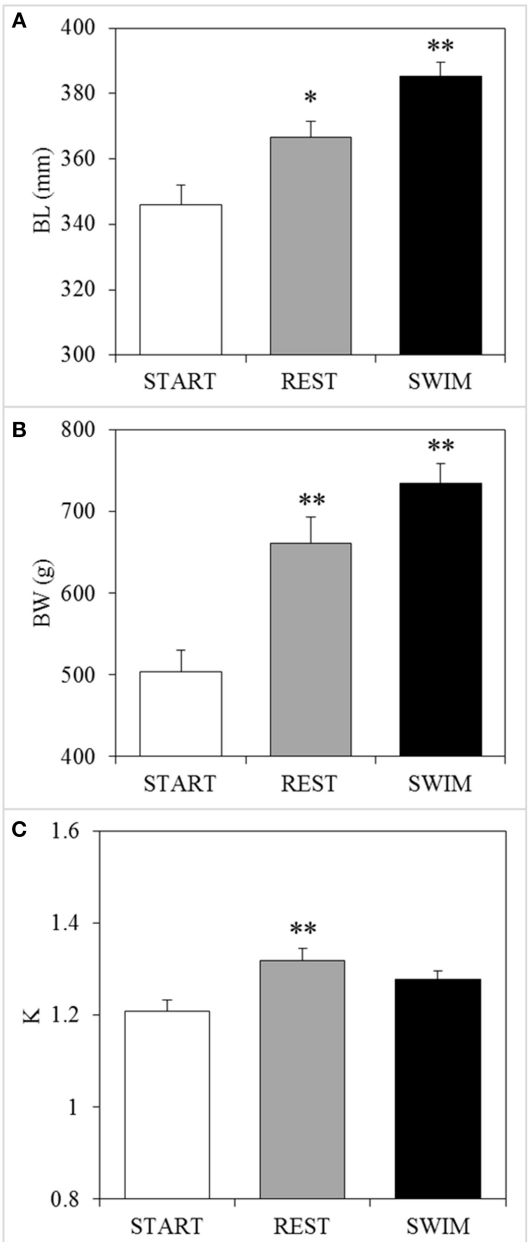

FIGURE 3 | Swimming-enhanced growth. After 18 days, swimmers ("SWIM," $n = 23$) showed **(A)** a significant 92% greater increase in body length (BL) and **(B)** a significant 46% greater increase in body weight (BW) as compared to resters ("REST," $n = 23$), while **(C)** Fulton's condition factor (K) was not different between the two groups. Asterisks above REST-bars indicate a significant difference (*$P < 0.05$; **$P < 0.01$) between the REST group and the START-group ($n = 10$: "time"-effects), while asterisks above SWIM-bars indicate significant differences between swimmers and resters ($n = 23$: "treatment"-effects).

Table 3 | Effects of performing sustained exercise for 18 days on heart weight and blood flow.

Parameters	START	REST	SWIM
HW (g)	0.95 ± 0.05	1.21 ± 0.06	1.22 ± 0.06
BF (mL min^{-1})		34 ± 3	**44 ± 3**

The START group ($n = 10$; TL = 346 ± 6 mm, BW = 504 ± 27 g) was sampled before the swim trial commenced. Fish swam ("SWIM," $n = 23$; TL = 385 ± 4 mm, BW = 735 ± 23 g) at 2.46 BL s^{-1} or were allowed to perform spontaneous movement ("REST," $n = 23$; TL = 367 ± 4 mm, BW = 661 ± 32 g) for 18 days. Hearts of 10 fish per group were weighted (HW). Blood flow in the ventral artery (BF) was measured in 13 resters and 13 swimmers. Parameters were statistically tested. Swimmers had a significant ($P < 0.05$) higher blood flow than resters, detected using pairwise ANCOVA with BW as cofactor and indicated in bold.

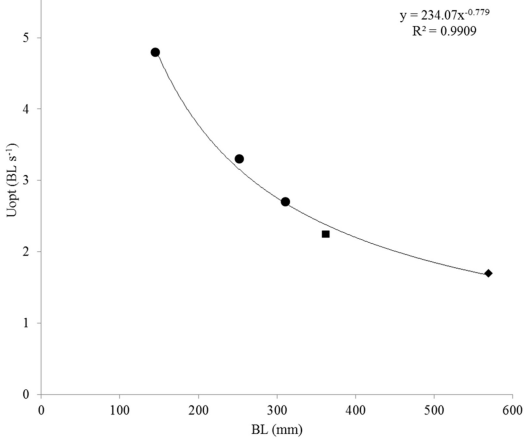

FIGURE 4 | Optimal swimming speed U_{opt} (in body length per second BL s^{-1}) in relation to size in BL. Shown are the three U_{opt}-values obtained in this study (circles) of group 1 (4.85 BL s^{-1}), group 2 (3.25 BL s^{-1}) and group 3 (2.73 BL s^{-1}); the U_{opt} (2.25 BL s^{-1}) of yellowtail kingfish of size 362 ± 15 mm, 699 ± 39 g as reported by Brown et al. (2011; square) and the U_{opt} (1.7 BL s^{-1}) of yellowtail kingfish of size 569 ± 26 mm, 2.35 ± 0.31 kg as reported by Clark and Seymour (2006; diamond). Best of fit is a power function: $y = 234.07x^{-0.779}$ ($R^2 = 0.9909$). Note that BL for fish of the study by Brown et al. (2011; square) is in fork length, adding 30–40 mm to estimate the TL-values leads to a perfect fit of this data point on the trend line.

Seymour, 2006; Petersen et al., 2011). When standardized for weight, swimmers in this experiment showed an average blood flow of 59 mL min^{-1} kg^{-1}, which approximates the results of Clark and Seymour (2006), who measured a blood flow of 50 mL min^{-1} kg^{-1} in yellowtails using silastic Doppler flow cuffs. This similarity between the blood flows obtained by an ADV vs. flow cuffs supports the potential of ADVs as alternative method for blood flow measurement, although more comparative studies need to be performed to fully assess the ADV's potential. The use of ultrasound techniques in teleosts is advancing rapidly: recently Guitreau et al. (2012) used ultrasound imaging to visualize the ovaries of submersed, non-anesthetized, unrestrained catfish. If protocols are optimized to allow the use of ADVs on non-anesthetized, submerged fish, e.g., by using water as a ultrasound transducer medium, the use of ADVs might pose a non-invasive, low-stress alternative to currently used surgical methods.

Vectrino measurements along the x-, y-, and z-axis in the newly designed swim-flume with motor-driven impeller showed a rather uniform flow in the swimming compartment in the applied configuration. When uniformity in flow is lacking, turbulence may have serious impact on the energy expenditure of swimming fish (Liao and Cotel, 2013). For example, fish swimming in vortices downstream of bluff bodies showed reduced muscle activity (Liao et al., 2013), while irregular turbulence has been shown to be detrimental to swimming fish, leading to increased energy expenditure on stability requirements in complex flows (Lupandin et al., 2000; Enders et al., 2003; Liao and Cotel, 2013). In our study, turbulence intensity in the swimming compartment of the flume was 0.08, which is considered low (Lupandin et al., 2000; Pavlov and Skorobogatov, 2009), and should therefore not disturb the optimal swimming economy of the fish in the experimental setup.

The created flow forced fish to swim continuously and in a sustainable way. Fish in the resting compartment showed spontaneous swimming activity, like being performed in tanks at the farm. Vectrino flow measurements in three tanks on the farm showed that fish of 600–1800 g were subjected to flows of 0.19–0.34 m s^{-1}, corresponding to ∼0.62–0.86 BL s^{-1}. Fish swam generally slightly faster than the flow indicating that the preferred swimming speeds were higher. Still, the preferred swimming speeds for the yellowtails under these conditions were not much higher than 1 BL s^{-1} and did by far not approach the optimal swimming speeds, contrary to a study on brook charr using a tilted raceway where preferred and optimal swimming speeds were found similar (Tudorache et al., 2011).

CONCLUSIONS

Our study has delivered a formula to calculate optimal swimming speeds for juvenile yellowtail kingfish over a size range of 145–311 mm BL, and together with data from literature (Clark and Seymour, 2006; Brown et al., 2011) up to 569 mm BL: U_{opt} (BL s^{-1}) = 234.07(BL)$^{-0.779}$ (R^2 = 0.9909). The formula was used to execute an experimental scale training experiment of 18 days. Results show that forced sustained exercise at optimal swimming speeds leads to a 92% greater increase in BL and 46% greater increase in BW, an exercise-induced growth stimulation that is much more pronounced than the 10% growth stimulation shown earlier for *S. lalandi* by Brown et al. (2011) and that is similar to the optimal growth stimulation for much smaller *S. quinqueradiata* juveniles (Yogata and Oku, 2000). The applied optimal swimming speed for the experimental fish in this study was 2.46 BL/s (0.85 m/s), much higher than the 0.75 BL s^{-1} at which Brown et al. (2011) found the highest growth rates, and also higher than the 1.5–2.0 BL s^{-1} as applied by Yogata and Oku (2000). Moreover, in this study non-swimming and swimming fish were given equal feeding rations (satiation for non-swimmers and restriction for swimmers) so that no differences in feed intake existed between both groups. The difference in growth is reflected by lower FCR for swimmers than for non-swimmers and is thus caused by higher feeding efficiency. It can be expected that when also swimmers are fed until satiation, exercise-enhanced growth will be even more pronounced. Exercise-enhanced growth is accompanied by higher blood flow in the ventral aorta, thus by increased cardiac output, a necessary adaptation in the aerobic phenotype to supply increased oxygen demands.

PERSPECTIVES

The outcomes of this study on experimental scale will need to be validated in an industrial scale setting where several aspects may differ from the experimental situation (densities, feeding conditions, water quality parameters, etc.). One aspect that will be different is that not a swim-flume will be applied but tanks or perhaps raceways as part of RAS having very different hydraulics. It will be challenging to create the flows that are required to induce optimal swimming, especially for the larger sized fish (>500 mm). Flows could be created by using motor-driven impellers like in this study, pumps or gravity, but perhaps a good alternative may be a device that exploits the optomotor response to encourage exercise, like the Optoswim concept (Herbert et al., 2011; reviewed by Herbert, 2013). When validated, implementation of optimal swimming regimes in yellowtail aquaculture, particularly in on-land RAS, will lead to a much faster production cycle. Moreover, increased production by exercise will most probably be accompanied by improved health and welfare aspects.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Arjan P. Palstra, Kees Kloet. Performed the experiments: Arjan P. Palstra, Daan Mes, Kasper Kusters, Jonathan A. C. Roques. Analyzed the data: Arjan P. Palstra, Daan Mes, Kasper Kusters, Jonathan A. C. Roques. Wrote the paper: Arjan P. Palstra, Daan Mes, Kasper Kusters, Gert Flik, Robbert J. W. Blonk.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fphys.2014.00506/abstract

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No evidence for a bioenergetic advantage from forced swimming in rainbow trout under a restrictive feeding regime

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Sustained swimming at moderate speeds is considered beneficial in terms of the productive performance of salmonids, but the causative mechanisms have yet to be unequivocally established. In the present study, the effects of moderate exercise on the bioenergetics of rainbow trout were assessed during a 15 week growth experiment, in which fish were reared at three different current speeds: 1 BL s^{-1}, 0.5 BL s^{-1} and still water ($\approx$ 0 BL s^{-1}). Randomly selected groups of 100 fish were distributed among twelve 600 L tanks and maintained on a restricted diet regime. Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated from weight and length measurements every 3 weeks. Routine metabolic rate (RMR) was measured every hour as rate of oxygen consumption in the tanks, and was positively correlated with swimming speed. Total ammonia nitrogen (TAN) excretion rates showed a tendency to decrease with increasing swimming speeds, yet neither they nor the resulting nitrogen quotients (NQ) indicated that swimming significantly reduced the fraction of dietary protein used to fuel metabolism. Energetic budgets revealed a positive correlation between energy expenditure and the current speed at which fish were reared, fish that were forced to swim and were fed restrictively consequentially had poorer growth and feed utilization. The results show that for rainbow trout, water current can negatively affect growth despite promoting minor positive changes in substrate utilization. We hypothesize that this may be the result of either a limited dietary energy supply from diet restriction being insufficient for both covering the extra costs of swimming and supporting enhanced growth.

Keywords: water current, feed conversion, oxygen consumption, nitrogen excretion, swimming, metabolic rate, fuel use

INTRODUCTION

The use of a moderate water current, to elicit a low level of sustained aerobic exercise, can have beneficial effects on several parameters of productivity and welfare of farmed salmonids (McKenzie et al., 2012; Davison and Herbert, 2013). Swimming induced benefits can be divided between physical effects of water current and derived behavioral changes as well as direct physiological benefits from swimming. Salmonids tend to exhibit schooling behavior when subjected to a water current, resulting in less agonistic interactions, reduced hierarchy formation, and lower stress levels (Christiansen and Jobling, 1990; Adams et al., 1995; Brännäs, 2009). Regardless of schooling, dominant fish may display less aggression to compensate for the higher energetic costs of swimming, which in turn increases the opportunities for subordinate individuals to feed (Christiansen and Jobling, 1990; Davison, 1997), and promotes growth by lowering stress levels in all categories of fish (Adams et al., 1995; Davison, 1997). While fish in schools obtain hydrodynamic benefits and swim steadily (East and Magnan, 1987), fish kept in still water display a much higher degree of spontaneous or erratic activity (Brännäs, 2009), with frequent changes in speed, acceleration, turning rate, and direction, a pattern that can be 2.5 to 6-fold more costly in energetic terms (Krohn and Boisclair, 1994; Steinhausen et al., 2010).

Cost of transport is often used as an argument for the benefits of swimming, regardless of the need for transportation. From a purely bioenergetic perspective, there can be no doubt that swimming is more costly than non-swimming. Still, evidence for enhanced growth performance (East and Magnan, 1987; Houlihan and Laurent, 1987; Jobling et al., 1993; Jørgensen and Jobling, 1993; Davison, 1997) suggests that the additional costs of swimming are more than met by compensatory gains. The mechanisms underlying such effects seem to result from a number of physiological changes induced by the presence of water current. Exercise can influence body composition through changes in protein and lipid deposition, although results from various studies are sometimes contradictory (Nahhas et al., 1982; East and Magnan, 1987; Houlihan and Laurent, 1987; Christiansen et al., 1989; Davison, 1997). Swimming may cause a reduction in the cost of living through changes in standard metabolic rate (Skov et al., 2011) while increased levels of circulating growth hormone and insulin-like growth factor (Sumpter, 1992; Davison, 1997; Deschamps et al., 2009) may influence protein turnover. It is known that fish peripheral tissues have a poor ability to clear a

glucose load from circulation, which has led some authors to refer to teleost fish as being glucose intolerant (Moon, 2001). However, exercise has been shown to have stimulatory effects on glucose utilization in rainbow trout red muscle (West et al., 1993; Felip et al., 2012). This is achieved in part by an increased glucose uptake by muscle cells (Felip et al., 2012) mediated by increased AMPK (Magnoni et al., 2012, 2014). Although the overall contribution from glucose in oxidative processes remains low, it could represent an avenue of improved energetic efficiency, since the extra costs of swimming could be counterbalanced by the efficient utilization of a fuel source that would be otherwise physiologically unavailable.

The purpose of the present study was to investigate the effects of water current on the growth performance and bioenergetics of rainbow trout maintained on a restricted diet, as a way to better expose the effects of exercise and prevent that differences in energy requirements be compensated and masked by an increased or differences in feed intake. Three different regimes were tested: a still water control group, a 0.5 body length per second intermediate current speed group, and a 1 body length per second group. The selected temperature was 15°C, considered to be the optimum temperature for growth in rainbow trout (Sumpter, 1992). By use of a rearing tank system that allowed instantaneous measurements of oxygen uptake, any differences in growth rate or feed conversion ratio (FCR) could be interpreted in the light of the relative energetic efficiency of the fish at different conditions. Energetic budgets were calculated based on values of feed intake and routine metabolic rates. In order to asses any correlation between exercise regime and protein retention, rates of ammonia excretion were measured and related to oxygen consumption in order to detect any differences in the rate of amino acid deamination.

MATERIALS AND METHODS

ANIMAL HUSBANDRY

Fish were obtained from a commercial fish farm (Funderholme Dambrug) and quarantined for 2 weeks in 15ppt sea water. Following quarantine, fish were randomly distributed in groups of 100 individuals among twelve circular polyethylene tanks with a water volume around 600 L, identical to those previously described by Larsen et al. (2012). Each tank had an internal cylindrical PVC column, creating a circular canal within the tank in which water could circulate. The tanks were connected to a common water supply within a recirculation bio-filtered system that delivered a 50 L min^{-1} flow of aerated freshwater at a constant temperature of 15°C to each tank by means of a centrifugal pump (Grundfos TP 25-90/2, Grundfos DK A/S, Bjerringbro, Denmark) fitted to each tank. Water quality parameters (NO_3^-, NO_2^-, NH_3/NH_4^+, pH) were monitored daily and did not exceed safe levels throughout the course of the study (NO_3^- <100 mg l^{-1}, NO_2^- 0–1 mg l^{-1}, total ammonia NH_3/NH_4^+ 0–1 mg l^{-1}). The inlet consisted of a vertical PVC pipe with a row of apertures fixed to the inner wall of the tank. The speed of the current could be controlled through the selection of aperture size and number and the direction at which the apertures of the inlet pipe were oriented relatively to the swimming canal. Water flow was adjusted in each tank so that three groups of four tanks were set with different current velocities while receiving the same volume of water over time. The different rearing conditions used were: no current (O); low current (LC) at 0.5 body lengths per second (BL s^{-1}); and high current (HC) at 1 BL s^{-1}. The velocity of the water current was measured twice per week using a propeller flow-meter (OTT Hydrometrie Z30, Germany) in the center of the swimming canal at three different depths. Minor adjustments were made by changes in water flow to the tank, while larger adjustments over time (as fish grew) were achieved by changing to inlet pipes with smaller apertures. In tanks with no water current the vertical inlet pipe was oriented so that the formation of a circular flow pattern never exceeded 0.1 BL s^{-1}, while water exchange were maintained at similar rates as in the other tanks.

Photoperiod was maintained at 14 h: 10 h light: dark (lights on at 7:00) throughout the experiment. Feeding was delivered by automatic belt feeders for periods lasting 8 h (between 8:00 and 16:00). The feeding regime was set up from Rasmussen and From's (1991) growth model based upon energy flow and partitioning parameters estimated from tank experiments with rainbow trout. This model defines feeding level f as the fraction eaten of the maximum quantity which could be eaten ($0 \leq f \leq 1$). The feeding level chosen for the growth trial was 0.84, corresponding to an initial daily ration of 1.3% of the estimated tank biomass and gradually reduced to a more restricted regime of 0.9% of the biomass. The feed consisted of 3 mm and later 4.5 mm extruded pellets (42–47% protein, 28–32% fat, 12–13% carbohydrate; EFICO Enviro 920, Biomar A/S, Brande, Denmark). The transition from 3 mm to 4.5 mm pellets took place between days 27 and 31 in a progressive way, with increments of 20% day^{-1} of the bigger sized pellets. Feces and debris were removed through a central drain in the bottom of the tank connected to a swirl separator, and any uneaten pellets were deducted from the daily ration. All use of animals for these experiments was in accordance with Danish and EU legislation.

GROWTH PERFORMANCE

Overall growth performance was calculated from a 15 week period (95 growth days), where fish mass and length were measured at five 21-day intervals, each consisting of 19 feeding days followed by 2 full days of fasting (the last interval had two extra days). Measurements were performed on day 21 and feeding was resumed the following day. Prior to manipulation, the fish were anesthetized with 2-phenoxyethanol (0.5 ml l^{-1}). The total biomass of each tank was measured, and the fish were counted to derive the mean mass for the fish in each tank. Photographs were used to measure the fork-length, with the aid of the software IrfanView 4.32. By the end of each measuring procedure the fish were returned to their tanks and current speed was adjusted according to the new mean body length for each tank. The total biomass in each tank was used to calculate the daily amount of feed for the following 19-day feeding period.

The specific growth rate (SGR) for each feeding period was calculated per tank on the total biomass, as:

$$SGR = 100 \times \left(\frac{\ln final\ biomass - \ln initial\ biomass}{number\ of\ days} \right) \quad (1)$$

Only feeding days were included in the calculation of SGR and the weights of fish that died during the period were included.

The FCR was calculated per tank as:

$$FCR = \frac{feed\ intake}{biomass\ increase} \quad (2)$$

The mean of the SGR and FCR values from all five intervals was used as the overall SGR and FCR.

ENERGETIC BUDGETS

Energetic budgets were calculated as previously described by Larsen et al. (2012) and McKenzie et al. (2012) for periods of similar growth for all treatment groups. Mean fish mass was calculated from the total biomass and number of individuals in each tank at each weighing-day and plotted against time to fit exponential growth curves for the entire growth trial. The growth curves were then used to identify periods when the fish had similar mean mass in all tanks. The selected time frame corresponds to the period when the fish grew from 250 to 350 g. For each of these days, feed intake and oxygen consumption were used to calculate several energetic parameters (see **Figure 2**).

STANDARD METABOLIC RATE

In order to assess the amount of energy required for maintenance by the fish from each current speed, the resting oxygen consumption was determined using computerized intermittent flow through respirometry (Steffensen, 1989; Skov et al., 2011). During a period of 2 weeks immediately after the conclusion of the growth trial, four individuals were successively sampled from each tank, with 24 h between one tank and the next (mean body mass for each group, grams ± SE: HC 411 ± 19; LC 400 ± 17; OC 402 ± 16; N=48 (16 per water current). Prior to experimentation, feeding was suspended for 48 h in the tank to be sampled, and resumed once fish had been removed. The fish were netted from the tank, weighed and transferred to 8.66 L Plexiglas respirometers immersed in a 60 L bath at 15°C, and covered with a sheet of black opaque plastic to prevent any external visual disturbance. Aerated and UV treated (9 W UV-C, AquaCristal GmbH, Neuhofen, Germany) water was recirculated between the experimental setup and a 600 L reservoir (40% replacement by volume daily) via a trickling filter. Oxygen concentration in the water was measured every second using fiber optic oxygen sensors (Fibox 3, Precision Sensing GmbH, Regensburg, Germany) collected by automated respirometry software (AutoResp, Loligo Systems, Tjele, Denmark). Fish were allowed to acclimate to the novel conditions overnight, and then oxygen consumption measurements were performed in 15 min cycles comprising an 8 min flushing period, a 1 min waiting period and a 6 min measurement period.

The software used the slope of oxygen decline during the measurement period to calculate the oxygen consumption as mg O_2 kg^{-1} h^{-1}. These data were sorted in 10 mg intervals to create a frequency distribution, and standard metabolic rate (SMR) was calculated as described previously (Skov et al., 2011).

ROUTINE METABOLIC RATE

The routine metabolic rate (RMR) of the fish was measured every hour as rate of oxygen uptake in each tank, using automated stop-flow respirometry (McKenzie et al., 2007, 2012). Tanks in the system alternated between periods of flow-through and recirculation without input of aerated filtrated water. Once per hour on the hour, a 3 way actuater (ER20, Valpes, Moirans, France) switched water flow to recirculation for a period of 10 min. During this time, the decline in water oxygen concentration was measured by oxygen electrodes (Oxyguard Standard, Oxyguard International A/S, Birkerød, Denmark) and recorded on a PLC data logger every 20 s during the periods where there was no renewal of water in the tanks. An emergency oxygen release system was present in each tank, to ensure that water SO_2 never fell below 70% at any time.

The data was sorted per tank in daily groups of 24 h and linear regression was performed on the decline in oxygen concentration. The gradient of oxygen decline, the total biomass of fish, and the total volume of water were then used to calculate oxygen consumption (MO_2; mmol kg^{-1} h^{-1}) by the fish in each tank.

The total biomass of the fish in each tank on each day was estimated from the SGR calculated for each 21-day period and the biomass measured at the start of the period, using:

$$\ln biomass_{dayX} = \frac{SGR}{100} + \ln biomass_{day(X-1)} \quad (3)$$

where $biomass_{dayX}$ = biomass on day of interest.

For comparing MO over the selected range of body masses (250–350 g), the data was made mass-independent by standardizing to a body mass of 300 g, using the equation:

$$MO_{2(300)} = MO_{2(m)} \times \left(\frac{m}{300}\right)^{1-A} \quad (4)$$

where $MO_{2(m)}$ = oxygen consumption for a fish with a body mass m; A = allometric exponent describing the relationship between metabolic rate and body mass. A value of 0.8 was used, as in previous studies (Skov et al., 2011; McKenzie et al., 2012). This exponent is generally accepted as reasonably accurate estimator of the change in metabolic rate with size of several fish species, including rainbow trout (Bureau et al., 2002).

The hourly measures of MO_2 during this period were summed to obtain the metabolic rate for the entire day and the average daily MO_2 among tanks with the same current speed used for comparison between the different treatments. Furthermore, circadian rhythms in oxygen consumption were revealed over the 24 h cycle and minimum and maximum MO_2 were found for each group.

Due to a failure of an O_2 probe, the oxygen consumption measurements from one tank in the O group were discarded.

AMMONIA EXCRETION AND PROTEIN USE

Water samples were collected on days 78, 80, and 82, for measurement of total ammonia nitrogen (TAN) excretion. Ammonia-N excretion (MNH_3-N; mmol kg^{-1} h^{-1}) was then related to the consumption of oxygen during the same period to calculate the extent of protein use, given by the nitrogen quotient (NQ).

The sampling procedure was automated and occurred simultaneously in the 12 rearing tanks, starting at 0:00 of each sampling day, and was repeated every 4 h corresponding to a total of 6 samplings per day. Twelve water pumps (Compact 300, Eheim, Germany) drew water from the tanks at the start and at the end of the 10 min periods of closed recirculation (6 s sampling, 0.5 l volume). Immediately after, 15 mL subsamples were centrifuged for 10 min at 3000 rpm, 0°C, and frozen at −20°C for later analysis. The increase in TAN content from initial to final samples corresponded to the excretion of ammonia in the tank.

Ammonia concentrations on the water samples were determined in duplicates using a spectrophotometric method based on the Danish standards for water analysis (DS, 1975).

The NQ was determined for each tank at each time as:

$$NQ = \frac{MN}{MO_2} \quad (5)$$

where MN is total nitrogen excretion and was estimated directly from MNNH_3-N, based on the work by Kajimura et al. (2004), who found that ammonia-N corresponds to 53–68% of the total nitrogen waste but, since the contribution of non-oxidized N-compounds was found to be on average 14%, this means that ammonia-N actually corresponds to 62–79% of the total pool of oxidized N-products. As the ammonia-N contribution is typically higher at low rations (Kajimura et al., 2004), the present study will use a NH_3-N contribution of 79%. Hence, the new $MN_{(oxidized)}$ was calculated from the measured MNH_3-N:

$$MN_{(oxidized)} = \frac{MNH_3\text{–}N}{79} \times 100 \quad (6)$$

The result was divided by MO_2 measured in the same period to obtain NQ.

The percentage use of protein to fuel metabolism was calculated by the ratio of NQ and 0.27, which represents the condition in which aerobic respiration is fuelled entirely by protein (Van Den Thillart and Kesbeke, 1978), and comparisons were made between the different treatments.

DATA ANALYSIS AND STATISTICS

Statistics were performed with SigmaPlot 11.0. The data were examined using One-Way analysis of variance (ANOVA). Holm-Sidak *post-hoc* tests were used to identify where any significant differences in an ANOVA had occurred. In all cases, $P < 0.05$ was the chosen level for statistical significance. All data are presented as mean values ± SE.

RESULTS

GROWTH PERFORMANCE

The mean mass of fish (± SE) at the beginning of the growth study was: 110.7 ± 1.3 (O), 110.5 ± 1.7 (LC) and 112.7 ± 1.1 (HC) grams, with no statistical differences between the three experimental groups. At the end of the growth period, the mean mass of fish was: 376.8 ± 4.3 (O), 373.5 ± 4.2 (LC) and 349.8 ± 8.6 (HC). There were significant differences between mean SGR calculated for the entire growth period (**Figure 1A**), overall being significantly lower in HC than in LC and in O. There were no differences in mean SGR between LC and O. The mean FCR was significantly higher for the HC group compared to the LC and O. (**Figure 1B**). There were no differences in mean FCR between LC and O. No differences in condition factor were observed between treatments (**Figure 1C**).

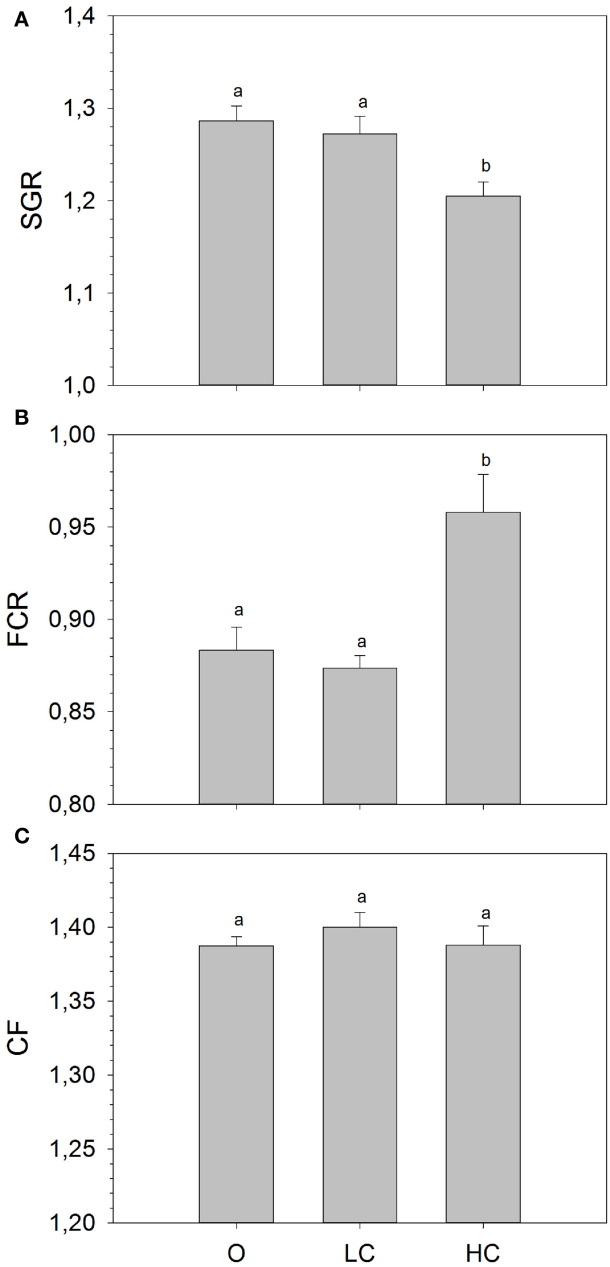

FIGURE 1 | (A) Specific growth rate (SGR), **(B)** Feed conversion ratio (FCR), and **(C)** Condition factor (CF) calculated for the entire duration of the growth trial. High current, HC = 1 BL s^{-1}; low current, LC = 0.5 BL s^{-1}; and O = no current (mean ± SE, $n = 4$). Different superscripts denote significant differences between treatments.

All water current regimes displayed clear circadian cycles with large increases in MO_2 during feeding hours and lower consumption levels during night time (**Figure 2**). There were no significant

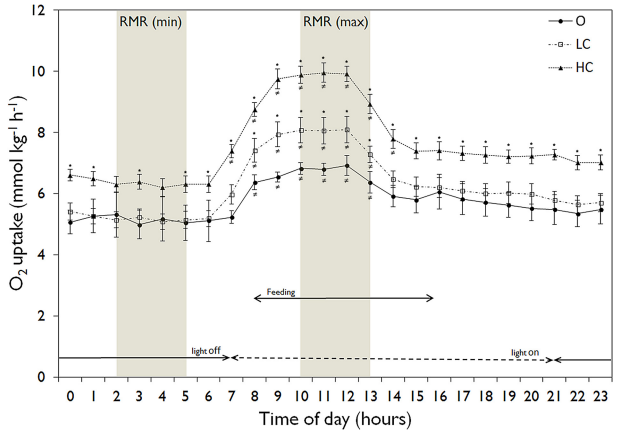

FIGURE 2 | Daily patterns of mean (± SE) hourly routine metabolic rate (RMR, normalized to 300 g) in the three experimental groups. High current, HC = 1 BL s^{-1}; low current, LC = 0.5 BL s^{-1}; and O = no current ($n = 4$ in HC and LC; $n = 3$ in O). Light conditions and feeding hours indicated by arrows. The shaded areas show the time points corresponding to the minimum and maximum values for RMR. An asterisk denotes significant difference from corresponding value for the O group, while a not-equal to sign denotes a significant difference from minimum RMR values.

differences in SMR between current groups (**Figure 3A**). Daily average RMR was significantly higher in the HC group, compared to the LC and O group (**Figures 2**, **3B**). The minimum (RMR_{MIN}, **Figure 3C**) and maximum (RMR_{MAX}, **Figure 3D**) RMR values occurred between 03:00 (O) and 04:00 (LC and HC) and between 11:00 (HC) and 12:00 (LC and O) respectively. For both minimum and maximum RMR values, the HC group was significantly higher than the O and LC groups.

ENERGETIC BUDGETS

The energetic budgets for each experimental group during an increase in mean individual body mass from approximately 250–350 g is shown in **Table 1**.

As the mean daily feed intake (g kg^{-1} day^{-1} ± SE) in this period was the same in all groups: 10.1 ± 0.04 (O), 10.0 ± 0.02 (LC), and 10.1 ± 0.04 (HC); this translated into a greater number of feeding days for the HC group to achieve a 100 g body mass increase. Feed loss was negligible in all tanks, a consequence of the restricted diet regime. The SGR calculated for this interval was significantly lower in HC than in LC and in O. Oxygen consumption in the selected period, and therefore total energy dissipation for metabolism, was significantly higher in HC than in LC and in O. Regarding the proportion of energy used relatively to the energy ingested, HC fish spent a significantly higher amount of energy than LC and O. The higher rate of energy utilization in the HC group meant that a significantly smaller proportion of the energy intake was apparently retained for allocation toward somatic growth. The gross cost of growth, i.e., the amount of energy required per gram of mass gained, was therefore higher in HC group compared to the other groups.

AMMONIA EXCRETION AND PROTEIN USE

The rate of TAN excretion over the six daily periods of measurement followed a similar circadian pattern as the oxygen consumption (**Figure 4**). All groups displayed a diurnal low at 8:00, immediately before the onset of feeding, after which TAN excretion rates began to increase. Each group showed a different progression in the three measuring periods after the onset of feeding: LC remained stable ∼0.7 mmol kg^{-1} h^{-1}, O steadily increased between 8:00 and 20:00, while HC peaked at 16:00 at ∼1.1 mmol kg^{-1} h^{-1} and then decreased at 20:00 to a similar rate as O (∼0.9 mmol kg^{-1} h^{-1}). The MNH_3-N (mmol kg^{-1} day^{-1}, mean ± SE) for HC, LC and O was: 19.8 ± 1.05, 15.3 ± 0.50, and 18.2 ± 1.29, respectively. MNH_3-N was significantly higher for HC than for LC.

The NQ assumes that the samples are representative of daily oxygen consumption and ammonia excretion. The mean (± SE) MO_2 and MNH_3 calculated from the average of the 3 days of sampling, as well as the calculated NQs, are given in **Table 2**. The oxygen consumption showed the same trend as throughout the remainder of the growth trial, with HC having a significantly higher rate than LC and O. The NQ for the O group was elevated, but not significantly so. The proportion of protein used for respiration was calculated from each NQ and are shown in **Table 2**.

DISCUSSION

GROWTH PERFORMANCE

Studies on Arctic charr (*Salvelinus alpinus* Linnaeus, 1758) (Christiansen et al., 1989, 1992; Christiansen and Jobling, 1990), Atlantic salmon (*Salmo salar* Linnaeus, 1758) (Totland et al., 1987; Jørgensen and Jobling, 1993; Jørgensen et al., 1996) and brook charr (*Salvelinus fontinalis* Mitchill, 1814) (East and Magnan, 1987) have obtained positive effects on growth and feed conversion ratio, when fish are reared at moderate current speeds and fed to satiation. The coincidence of U_{OPT} with optimal swimming speed for growth appears to be the case for many species, including the salmonids (Davison and Herbert, 2013), although for rainbow trout, research on exercise induced promotion of growth has not yielded unequivocal results. So the question is whether rainbow trout is the odd one out or whether other things factor in. In the present study, the highest water velocity used was 1 BL s^{-1} which is considered to be the most energetically efficient swimming speed for rainbow trout with a mass of ∼250 and reportedly the optimal for growth (Webb, 1971; Weihs, 1973; Walker and Emerson, 1978). The fish in this study were smaller than this size and were therefore in fact swimming well below their U_{OPT}, emphasizing the need to consider what the U_{OPT} is for the size of fish being used.

Houlihan and Laurent (1987) did obtain higher growth rates in trout reared at 12°C swimming at 1 BL s^{-1} compared to non-swimming fish. These fish were fed to satiation, with no further report on the specific feed intake. McKenzie et al. (2012) found no positive effects on growth or feed conversion when feeding was limited to 1% at 0.9 BL s^{-1} and 14°C.

The present study found a significant negative effect of exercise at a current of 1 BL s^{-1} on both SGR and feed conversion ratio. The fish were reared in circular tanks, which dictated a swimming pattern with a turning component included at all times rather than a simple straight line motion. At the same time, a circular swimming canal will dictate a velocity gradient from outer to inner wall, and a drag effect along the tank walls. Although

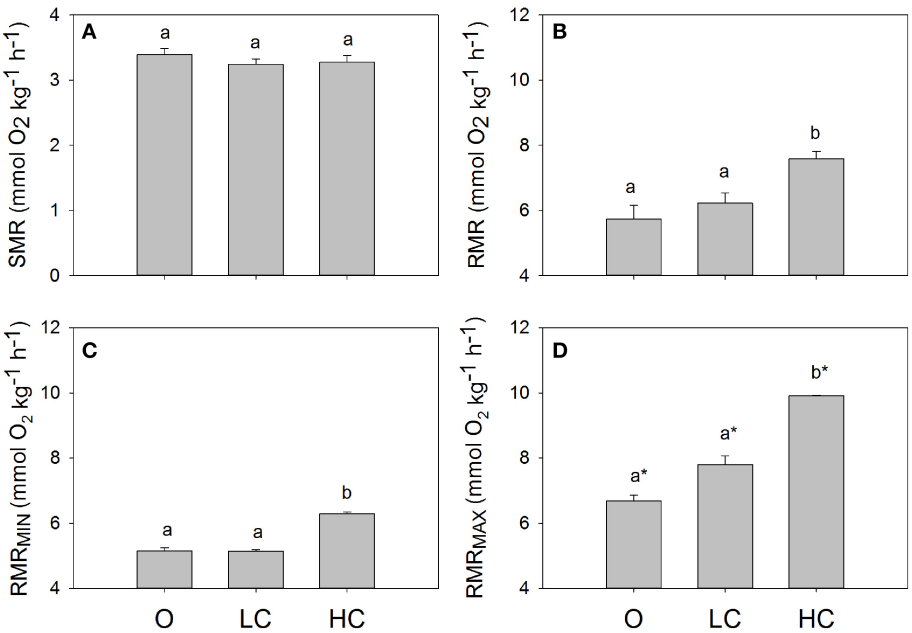

FIGURE 3 | Standard and routine metabolic rates for fish reared at different current speeds. (A) Standard metabolic rate, SMR, **(B)** daily mean, **(C)** minimum and **(D)** maximum routine metabolic rates, RMR. Superscripts indicate significant differences between treatments. High current, HC = 1 BL s^{-1}; low current, LC = 0.5 BL s^{-1}; and O = no current ($n = 4$ in HC and LC; $n = 3$ in O). Superscript letters indicate significant differences between treatments, asterisks in **(D)** indicate significant differences from minimum RMR values.

Table 1 | Oxygen consumption, ammonia excretion rates, and nitrogen quotients (NQ) for groups of fish subjected to different water current velocities.

	O	LC	HC
Oxygen consumption (mmol O_2 kg^{-1} day^{-1})	140.7 ± 12.3^a	158.0 ± 6.95^a	192.5 ± 7.14^b
Total ammonia nitrogen excretion (mmol Nkg^{-1} day^{-1})	20.8 ± 2.2	19.7 ± 2.3	22.7 ± 2.9
NQ	0.164 ± 0.006	0.126 ± 0.011	0.130 ± 0.019
%Protein use	60.8 ± 2.3	46.5 ± 4.1	48.1 ± 6.8

Values are mean (±SE) from 3 days of measurements (days 78, 80, and 82 of the growth trial). High current, HC = 1 BL s^{-1}; low current, LC = 0.5 BL s^{-1}; and O = no current (n = 4 in HC and LC; n = 3 in O). Superscripts indicate significant differences between treatments.

we did not systematically quantify these effects, any gradient or wall effect did not have any apparent effect, and fish positioned themselves evenly distributed in the water column and along the width of the canal. With the exception of Totland et al. (1987), all of the above mentioned studies employed circular rearing tanks, and the spatial properties of the rearing system seems an unlikely explanation for the differences in the results. Rather, the use of a restricted feeding regime may be an explanation for the poor SGR and FCRs obtained in this study, as was also observed in the study by McKenzie et al. (2012). When fish are fed *ad libitum*, it is likely that they are in energetic surplus (McKenzie et al., 2012), so that the increased cost of swimming does not limit growth. In that situation, the beneficial effects of water current, such as inhibition of aggressive behavior or erratic activity, can be of adequate magnitude to be reflected in growth.

ROUTINE METABOLISM

The rates and daily patterns of oxygen uptake were in line with previous investigations (McKenzie et al., 2012). There was a clear increase in routine activity when lights were turned on, that was reflected in the consumption of oxygen in the tanks. When feeding began, MO_2 furthermore increased. This postprandial increase in metabolic rate corresponds to the specific dynamic action (SDA) and is believed to be associated with digestion, absorption, and protein assimilation (Jobling and Davies, 1980; Alsop and Wood, 1997; Owen et al., 1998; Seth et al., 2009). The HC group had a markedly higher RMR throughout the whole day and on average for the entire experiment.

BIOENERGETIC BUDGETS

The lower SGR of the HC group resulted in an increase in the number of days required to achieve an average mass gain of 100 g. Since all treatments had a similar intake of dietary energy, and assuming that digestibility of nutrients was equal between treatments, the decrease in SGR can be assigned to the increase in energy expenditure on routine activity. These findings demonstrate that rainbow trout forced to swim at 1 BL s^{-1}, have a greater requirement for maintenance energy, which they must fuel by oxidizing a significantly higher fraction of their ingested energy at the cost of somatic growth. It is commonly accepted that exercising salmonids if fed to satiation, show faster growth than still water controls since they display an increase in appetite; however, when smaller rations are employed, growth rates are not

improved (Davison, 1989). In addition to increase feed intake, this improved growth appears to be also mediated by improved nutrient retention, as shown by Grisdale-Helland et al. (2013) who also observed higher maintenance energy requirements in Atlantic salmon swimming at speeds of 1.1 vs. 0.3 BL s^{-1}, that did not manifest in different growth rates despite being maintained on rations of 0.9% BM d^{-1}.

AMMONIA EXCRETION AND PROTEIN USE

Daily fluctuations in TAN excretion are linked to feeding and the maximum postprandial excretion, and are typically 30–67% higher than the daily mean (Thorarensen and Farrell, 2011), which is in line with the observations in the present study. The average daily ammonia excretion was significantly higher in HC than in LC. This shows that absolute rates of protein turnover increase with increasing current speeds. However, the fact that MNH_3-N in O was not significantly different from the groups with water current suggests that we should be cautious in the conclusions we draw.

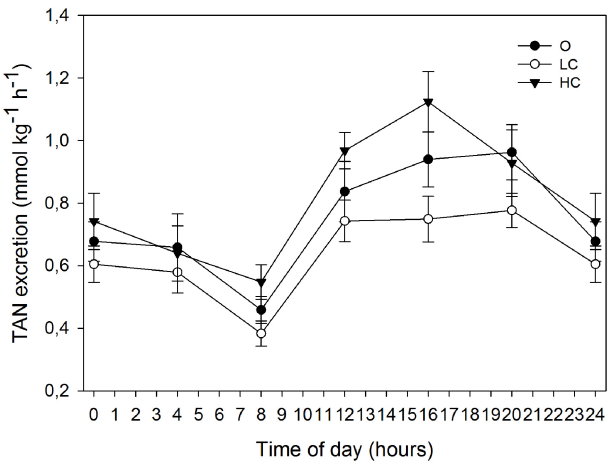

FIGURE 4 | Daily patterns of total ammonia nitrogen (TAN) excretion at different water current velocities. Values are mean (±SE) for 3 days of measurements (days 78, 80, and 82 of the growth trial). High current, HC = 1 BL s^{-1}; low current, LC = 0.5 BL s^{-1}; and O = no current ($n = 4$).

The NQ is a measure of protein utilization obtained from the relationship between oxygen consumption and nitrogenous waste excretion. The results of the present study show average rates of protein use to fuel metabolism is slightly but not significantly higher in the O group (∼60%) while the LC and HC groups used less than 50%. These tendencies in the NQ values are brought on by changes in MO_2 and less by MN. NQ values for fish are highly variable, ranging between 14% and 90% in different species (Van Waarde, 1983; Weber and Haman, 1996; Lauff and Wood, 1996b; McKenzie et al., 2007). In fasted salmonids protein has been estimated to contribute 14–36% but these values may increase up to close to 100% in actively feeding fish (Kajimura et al., 2004). The values found in this study are higher than those obtained by Lauff and Wood (1996a,b) in studies of instantaneous fuel usage during aerobic swimming in fasted juvenile rainbow trout (22–45% in untrained fish and 17–36% in fish trained at 2.1 BL s^{-1}), but are in agreement with the results obtained by Alsop and Wood (1997) in trout fed 1% body mass per day (∼40%) and fed to satiation (∼70%). The abovementioned studies employed an instantaneous approach based on respirometry and measurement of excreted ammonia-N and urea-N. However, the use of summed ammonia-N and urea-N is thought to underestimate the contribution of protein toward metabolism (Kajimura et al., 2004) because it does not consider other oxidized N-products resulting from protein catabolism. On the other hand, NQ based on total-N excretion may overestimate protein oxidation because total-N includes a significant proportion of non-oxidized N-products such as proteins excreted in mucus.

A complete analysis of fuel usage would require the measure of carbon dioxide excretion rates and calculation of respiratory quotients besides nitrogen quotients, and should be conducted on fed as well as fasted fish. A more detailed understanding of instantaneous fuel use is key to explaining exercise induced, non-feed intake related, effects on growth. While the present study does not allow a full discussion on the partition of metabolic fuels between

Table 2 | Energetic parameters in fish reared under three different current speeds, high current, HC = 1 BL s^{-1}; low current, LC = 0.5 BL s^{-1}; and O = no current (mean ± SE, $n = 4$, except for O, where $n = 3$ in O_2 consumed, E dissipated, E allocated and gross cost of growth).

	O	LC	HC
Mass gain (g $fish^{-1}$)	99.60 ± 0.85	101.1 ± 0.43	100.5 ± 0.52
Time required (days)	28.3 ± 0.48	28.8 ± 0.25	30.3 ± 0.48
SGR (% day^{-1})	1.18 ± 0.01[a]	1.18 ± 0.02[a]	1.12 ± 0.02[b]
Total feed intake (g kg^{-1})	285.0 ± 5.49[a]	288.6 ± 3.03[a]	304.3 ± 4.35[b]
Total E intake (kJ kg^{-1})	6981.3 ± 134.44[a]	7070.5 ± 74.16[ab]	7455.6 ± 106.54[b]
Total O_2 consumed (mmol kg^{-1})	3730.6 ± 273.11[a]	4153.5 ± 210.52[a]	5304.9 ± 182.70[b]
Total E dissipated for metabolism (kJ kg^{-1})	1622.8 ± 118.80[a]	1806.8 ± 91.58[a]	2307.6 ± 79.48[b]
%E dissipated for metabolism	23.6 ± 1.72[a]	25.6 ± 1.26[a]	30.9 ± 0.90[b]
%E allocated for growth	76.4 ± 1.72[a]	74.4 ± 1.26[a]	69.1 ± 0.90[b]
Total E allocated for growth (kJ kg^{-1})	5265.4 ± 166.70	5263.8 ± 105.29	5147.9 ± 91.81
Gross cost of growth (kJ g^{-1})	20.60 ± 0.16[a]	20.86 ± 0.30[a]	22.11 ± 0.28[b]

The parameters were calculated per tank (kg biomass), except for SGR and gross cost of growth (see below). Superscripts indicate significant differences between treatments.

protein, lipid and carbohydrates, the results indicate a trend for a decrease in the relative contribution of protein when a current is present. This suggests that there may be a decrease in protein catabolism during aerobic swimming, which is in accordance with the findings of Alsop and Wood (1997) and Lauff and Wood (1996a,b), however, it is evident that for trout and salmon this depends on several factors, in particular feed intake and dietary macronutrient content. This is exemplified by the work of Felip et al. (2012) who observed significant increases in nitrogen recovery in white and red muscle protein when rainbow trout were forced to swim while fed a diet with 30% digestible carbohydrate. This effect was brought on partly by the aforementioned increase in glucose oxidation, but also by an increased lipogenesis which was subsequently used to fuel swimming. When salmon are reared on commercial diets with lower levels of carbohydrate (e.g., 12–17%) this effect has not been observed.

CONCLUSION

The present study has contributed to illustrate the complexity of the physiological responses of rainbow trout to a moderate exercise. It seems that swimming against a current does not confer an energetic advantage *per se*; rather it may even negatively affect growth performance. The proposed explanation for these results is an exacerbation of the metabolic expenditure relative to the total energy available in exercised fish, possibly due to a restricted feeding regime. While imposing an exercise regime on fish carried a significant increase in metabolic cost, calculated nitrogen revealed a tendency toward a reduced relative use of protein to fuel metabolism in exercised fish. The use of lipid and carbohydrate to fuel exercise metabolism during fasting and feeding should be quantified as a logical next step. The results of this study also lend support to previous findings of a reduction in minimum metabolic requirements induced by exercise. This effect deserves further investigation.

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Accommodating the cost of growth and swimming in fish—the applicability of exercise-induced growth to juvenile hapuku (*Polyprion oxygeneios*)

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Induced-swimming can improve the growth and feed conversion efficiency of finfish aquaculture species, such as salmonids and *Seriola* sp., but some species, such as Atlantic cod, show no or a negative productivity response to exercise. As a possible explanation for these species-specific differences, a recent hypothesis proposed that the applicability of exercise training, as well as the exercise regime for optimal growth gain ($ER_{opt\ growth}$), was dependent upon the size of available aerobic metabolic scope (AMS). This study aimed to test this hypothesis by measuring the growth and swimming metabolism of hapuku, *Polyprion oxygeneios*, to different exercise regimes and then reconciling the metabolic costs of swimming and specific dynamic action (SDA) against AMS. Two 8-week growth trials were conducted with ERs of 0.0, 0.25, 0.5, 0.75, 1, and 1.5 body lengths per second (BL s^{-1}). Fish in the first trial showed a modest 4.8% increase in SGR over static controls in the region 0.5–0.75 BL s^{-1} whereas the fish in trial 2 showed no significant effect of ER on growth performance. Reconciling the SDA of hapuku with the metabolic costs of swimming showed that hapuku AMS is sufficient to support growth and swimming at all ERs. The current study therefore suggests that exercise-induced growth is independent of AMS and is driven by other factors.

Keywords: exercise training, swim-flume respirometry, aerobic metabolic scope, optimal swim speed, cost of transport

INTRODUCTION

There is ample evidence in the literature showing that induced swimming, or exercise training, can improve the growth and feed conversion efficiency of many species of farmed fish (Davison, 1989; Palstra and Planas, 2011; Davison and Herbert, 2013). Most of this evidence has been accumulated in the salmonid groups *Oncorhynchus* (Houlihan and Laurent, 1987; Aslop and Wood, 1997; Hernández et al., 2002), *Salmo* (Davison and Goldspink, 1977; Totland et al., 1987; Boesgaard et al., 1993) and *Salvelinus* (Leon, 1986; Christiansen et al., 1989; Christiansen and Jobling, 1990) but there are examples of exercise-induced growth from other groups, with species such as the striped bass *Morone saxatilis* (Young and Cech, 1993) and the yellowtail kingfish *Seriola lalandi* (Brown et al., 2011). The global aquaculture industry is expanding rapidly and the potential for continuous exercise to accelerate the growth of fish has direct application due to the potential for fast biomass gain, improved flesh quality and the flexibility of production it can provide. However, exercise-induced growth is often perceived as a paradoxical concept as it seems illogical that fish can expend considerable energy on exercise whilst also committing to the extra expense of accelerated growth. This view is reinforced by a number of studies showing that exercise has either nil, or only negative effects on the growth of fish such as the Atlantic cod *Gadus morhua* (Bjørnevik et al., 2003) and Chinook salmon *Oncorhynchus tshawytscha* (Kiessling et al., 1994). Therefore, to stand any chance of exploiting the economic gains of exercise-induced growth in aquaculture, an in-depth understanding of how fish balance the metabolic costs of growth and exercise needs to be ascertained, particularly in the case of information-poor species that are new to farming.

There has been a rekindled interest in the mechanisms and applicability of exercise-induced growth in recent years (Palstra and Planas, 2011) and new efforts have been made to predict the levels of exercise required for the best rate of growth in novel species using readily accessible measures of behavior and physiology (Davison and Herbert, 2013; Herbert, 2013). In particular, the aerobic metabolic scope (AMS) of fish and the speed where the energetic cost of transport (COT) is at its lowest, termed the optimal swimming speed (U_{opt}), appears to explain a significant proportion of the variation between different fish that show exercise-induced growth (Davison and Herbert, 2013). AMS is the difference between maintenance and maximal metabolic rates, and thus represents a physiological framework, within which non-maintenance physiological work operates (Jobling, 1994; Clark et al., 2013). In light of this belief, it has been suggested that a larger AMS better accommodates the energetic costs

of swimming in addition to other processes, such as protein synthesis associated with growth and feeding (von Herbing and White, 2002; Davison and Herbert, 2013). There are few experimental studies on this topic but the work of Owen (2001) on the European eel *Anguilla anguilla* appears to support this assertion. Indeed, where AMS was insufficient, or the costs of feeding (specific dynamic action, SDA) were deemed excessive, the swimming speed of eels were reduced to accommodate SDA as a form of energetic prioritization (Owen, 2001). On the basis of these observations, Davison and Herbert (2013) examined how the required exercise regime for optimal growth acceleration (termed $ER_{opt\ growth}$, in units of body lengths s^{-1}, BL s^{-1}) co-varied with AMS in a variety of well-studied species. As a positive but non-linear correlation was found between $ER_{opt\ growth}$ and AMS, Herbert (2013) proposed that AMS might have value in predicting the $ER_{opt\ growth}$ of novel fish species in which the effects of exercise have yet to be investigated. Of particular relevance to those species that show both a positive growth response to exercise and a direct relationship between U_{opt} and $ER_{opt\ growth}$ is the suggestion that that U_{opt} speeds are preferentially selected by some migratory fish across extended periods (Hinch and Rand, 2000; Tudorache et al., 2011). This would imply that, when swimming is required, a minimization of swimming costs per unit distance may allow for a greater proportion of available AMS to be allocated to somatic growth. Therefore, to summarize this collective background, fish with a sufficiently high AMS are expected to have the capacity to swim and grow fast at the same time (Davison and Herbert, 2013; Herbert, 2013) and, in this scenario, U_{opt} is also believed to predict the best flow regime (speed) for growth (Davison and Herbert, 2013).

In an attempt to test and validate the proposed models of Davison and Herbert (2013) and Herbert (2013), the exercise-induced growth performance of juvenile hapuku, *Polyprion oxygeneios*, a novel farmed finfish species from New Zealand, was quantified and compared against experimentally derived measures of AMS and U_{opt}. Specifically, if the AMS-$ER_{opt\ growth}$ model of Davison and Herbert (2013) is applicable to a wider range of species, then the predicted AMS value of hapuku at 17°C (300 mg O_2 kg^{-1} h^{-1} at 17°C, based on the data of Khan et al., 2014) is hypothesized to provide the metabolic capacity for optimal exercise-induced growth in the vicinity of ∼0.4–0.5 BL s^{-1} (Herbert, 2013). If exercise-induced growth is indeed observed in hapuku, U_{opt} and $ER_{opt\ growth}$ should also be relatively well matched (Davison and Herbert, 2013). As a further step in this validation and testing process, the metabolic costs of swimming at different speeds and the recently measured cost of SDA (Khan et al., in press), which is largely comprised of post-absorptive protein synthesis and growth (Secor, 2009; Seth et al., 2010) was also reconciled against the available AMS. This allowed for the experimental resolution of whether hapuku have metabolic capacity to accommodate swimming and the physiological costs associated with growth.

MATERIALS AND METHODS

SPECIMENS, TAGGING, AND GROWTH TRIALS

Two full- and half-sibling groups of ∼120 juvenile hapuku (*P. oxygeneios*, ∼8 months post-hatch, 240 in total) were used for growth trials at the NIWA Bream Bay Aquaculture Facility in Ruakaka, Northland, New Zealand. "Trial 1" fish (128.8 g ± 3.1 g) were hatched 12 weeks prior to "trial 2" fish (172.4 ± 4.5 g) and were also smaller at the start of the growth trials as they were 4 weeks younger at the point when they entered the experimental tanks. Both groups of fish were held at 17°C in larger 4 m^3 tanks prior to the start of both trials. To track the growth and performance attributes of individuals, all fish were tagged intraperitoneally with a 5 mm passive integrated transponder (PIT) under anesthesia (0.01 mL L^{-1} Aqui-S® followed by 0.3 mL L^{-1} 2-phenoxyethanol, standard facility practice). Specimens were treated with chloramine-T (0.005 mL L^{-1}) to prevent infection post-tagging (added to flowing tank water, standard facility practice). Any individuals that showed signs of infection were treated further with formalin (0.15 mL L^{-1}) or euthanized with an excessive dose of Aqui-S® (0.1 mL L^{-1}). Thereafter, two sequential and identical growth trials were conducted incorporating six different exercise regimes (ER, corresponding to six in-tank flow speeds of 0.0, 0.25, 0.5, 0.75, 1, and 1.5 body lengths per second, BL s^{-1}). Each of the two trials were conducted in six identical 1.6 m^3 circular tanks (560 mm water depth, 1900 mm diameter). All tanks were housed in a purpose-built building under ambient light conditions (11L: 13D) and supplied with fresh 1 μm filtered and UV-sterilized (ALX2/8, 150 mW s cm^{-2}, Davey Water Products, Australia) seawater at 17 ± 0.3°C. A continuous non-directional inflow of water (30 L min^{-1}) was present at the side of each tank and all tanks were central draining. Water flow around the tank was negligible in the 0.0 BL s^{-1} (control) tank but the remaining water flow ER treatments (i.e., 0.25, 0.5, 0.75, 1, and 1.5 BL s^{-1}) were maintained through the use of external water pumps (Leader® Ecopool 15, Leader Pumps, Italy) plumbed over the side of each tank via a 25 mm PVC intake and outlet. Pump outlets were connected to a spray bar at a water depth level of 100 mm from the surface and the spray bar extended 500 mm into the tank at a perpendicular angle. Water flow through the spray bars (and thus flow speed in the tanks) was controlled through a ball valve plumbed between the pumps and the spray bars. Flow speeds were set in the tanks by measuring water velocity (in m s^{-1}) 200 mm from the tank wall at 100 mm depth on the side directly opposite the spray bar with a Höntzsch® HFA anemometer (V 1.5, Höntzsch technologies, Waiblingen, Germany) and making the necessary correction to the flow of water according to the average body length of the fish at regular fortnightly intervals. Each tank had a single projection of PVC pipe (200 mm high, 100 mm diameter) off the floor, approximately half way between the wall and the center. They were entirely submerged, impossible to remove and created a small low-flow area in their wake. Water chemistry was checked regularly and remained at normal levels at all times throughout both trials.

The fish intended for trial 1 were anesthetized (as described above) in their pre-trial holding tank at 17 ± 0.3°C and their initial weight and length were measured. They were then divided randomly and evenly (∼20 per tank) into one of the six experimental tanks and allowed to recover for 4 h with no directional flow. Once swimming behavior appeared normal, flow speeds in the tanks were increased slowly toward one of the six exercise training speeds in BL s^{-1} according to the average BL of all

fish in each tank. All tanks were fed to satiation twice a day (at ∼0800 and 1600 h) for the following 12 days on Skretting Nova FF 5/7 mm pellets (Skretting, Australia, 50.0% protein, 17.0% lipid, digestible energy 18.6 MJ kg^{-1}). Any uneaten feed was recovered 15 min after feeding behavior had ceased (low tank densities allowed feeding behavior to be observed accurately by an observer). The weight of recovered feed was corrected for water absorption by a standard saturation factor (determined by soaking a known weight of feed pellets and then re-weighing, equating to 1.6 × dry weight at saturation). After 12 days all specimens were starved for 48 h and their weight and length recorded under anesthesia (as described above). Water speeds in each tank (other than the control) were then increased to match the increased length of the fish, in order to maintain treatments of 0.25, 0.5, 0.75, 1, and 1.5 BL s^{-1}. This was followed by another 12-day period on the same feeding regime. This cycle was repeated twice more to give a total of four 12-day feeding periods interspersed with assessments and adjustments to water speeds. All tanks were treated with Chloramine-T (0.005 mL L^{-1}) once per day for 3 days after any handling event and at least 3 h before feeding. There was no measureable difference in feeding behavior between days with Chloramine-T treatments and those without. To ensure ER regimes were maintained at a target level, water flow was checked daily at a position on the opposite side to the spray bar, and at regular spacing intervals around the tank weekly. The weight and length of each individual fish were recorded at the end of the trial under anesthesia (anesthetized as described above).

One week after the end of trial 1, trial 2 commenced and fish were treated in exactly the same way as trial 1 but with ER treatments (0.0, 0.25, 0.5, 0.75, 1, and 1.5 BL s^{-1}) randomly reassigned to one of the six tanks. The only other exception was a reduction in the number of fish per tank in the second trial (17 fish per tank in trial 2 vs. ∼20 per tank in trial 1) and therefore a difference in biomass density between trials (trial 1 = 1.99 ± 0.12 kg m^{-3}; trial 2 = 2.03 ± 0.22 kg m^{-3}).

Mass specific growth rate (SGR, % body weight day^{-1}) was calculated for each individual using the formula:

$$\mathrm{SGR} = (\ln m_2 - \ln m_1)/(t_2 - t_1) \times 100$$

where, m_1 is the initial weight at the start of the growth period t_1 and m_2 is the final weight at the end of the growth period t_2.

Feed conversion ratio (FCR), measured as the weight of dry feed intake (corrected for uneaten feed) per unit weight gain for the period, and was calculated for each tank using the following formula:

$$\mathrm{FCR} = \text{weight of dry feed consumed in tank/ wet weight gained in tank}$$

The initial and final condition factor (CF) of fish was also calculated using the formula:

$$\mathrm{CF} = \mathrm{mass}/\mathrm{length}^3 \times 100$$

The relative change in CF (ΔCF) over the course of the growth trials was then calculated as the difference between final and initial CF.

RESPIROMETRY

Swim flume respirometry was performed on fish from three ER treatments (0.0, 0.75, and 1.5 BL s^{-1}) to resolve the effect of exercise training on metabolic cost functions. Aside from understanding the potential metabolic effects of long-term exercise, this information was important for reconciling the cost of growth and swimming. All specimens were starved for 48 h prior to respirometry to remove any confounding effects of feeding on metabolic rate (Ross et al., 1992; Thuy et al., 2010). The mass specific rate of oxygen consumption (MO_2, mg O_2 kg^{-1} h^{-1}) was then determined from 24 fish from trial 2 (i.e., 8 fish from the 0.0, 0.75 and 1.5 BL s^{-1} ER groups) over a period of 30 days in the 38.4 L Brett-type swim flume respirometer described by Brown et al. (2011). The change in oxygen saturation in the respirometer was measured continuously using a Firesting® 2-channel oxygen meter (Pyroscience, Germany) connected to an oxy-dipping probe (Pyroscience, Germany) which was sealed into the respirometer in a position anterior to the swimming section. The respirometer was operated through a custom software interface which controlled water flow speed in the swimming section and the cycling of the flush, wait and measure periods (5, 1 and 4 min, respectively, 10 min total). MO_2 and its components were calculated using the same formulae as Brown et al. (2011).

After measuring the weight, length, depth, and width of fish [to compensate for the solid-blocking effect (Steffensen, 1989)], specimens were placed in the sealed swimming section of the respirometer (530 × 130 × 155 mm). This occurred at ∼1600 h and provided fish an overnight period of acclimation to the conditions of the respirometer with a low flow of water (0.25 BL s^{-1}) and with the system cycling automatically through a repeated series of flush, wait and measure. From 0800 the following day, a critical swimming speed (U_{crit}) test commenced where the flow speed inside the swimming section was increased by 0.25 BL s^{-1} every 30 min (i.e., after three 10 min flush-wait-measure cycles). This continued until 1/3 of the body was pressed up against the rear of the swimming section or erratic and non-directional burst activity was observed. Fish swimming behavior was monitored at all times with a CCD camera (KT & C 19 mm, Seoul, Korea) attached to an external monitor. After each experiment was complete, background oxygen consumption levels were measured without a fish and confirmed that bacterial respiration was essentially nil and negligible in all runs. All equipment was cleaned thoroughly between experiments with freshwater and a mild hypochlorite solution (0.005 g L^{-1}).

For each individual fish, critical swimming speed (U_{crit}) was calculated using the same formula as Brett (1964), Brown et al. (2011), and Yanase et al. (2012). The 15% quantile method of Chabot and Claireaux (2008) and Franklin et al. (2013) was used to obtain a near-resting value of MO_2 from overnight measures at 0.25 BL s^{-1} in order to remove erroneously-low vales associated with unusually weak oxygen probe signals. Thereafter, three MO_2 values obtained from each of the three flush-wait-measure cycles at each speed were averaged to resolve the relationship between swimming speed and MO_2 (Korsmeyer et al., 2002; Brown et al., 2011) at speeds that were considered to be exclusively aerobic, i.e., up to 2.5 BL s^{-1} (Roche et al., 2013). In order

to yield an estimate of standard metabolic rate ($MO_{2standard}$) for every individual fish, average MO_2 at all speeds was extrapolated back to 0.0 BL s^{-1} using an exponential regression function as used previously by other authors (Pettersson and Hedenström, 2000; Yanase et al., 2012) as power functions underestimated $MO_{2standard}$ values compared to other investigations on the same species (Khan et al., 2014, in press). Using all MO_2 values from the point that fish first entered the respirometer, MO_{2max} was calculated using the 99% quantile method of Khan et al. (2014) as this yielded higher, and produced less inter-individual variation, than MO_2 values at U_{crit}. AMS was calculated by subtracting $MO_{2standard}$ from MO_{2max} and the gross cost of transport (GCOT, mg O_2 kg^{-1} BL^{-1}) was calculated by dividing MO_2 by their corresponding swimming velocity (BL s^{-1}).

STATISTICAL ANALYSES

The data relating the effect of ER treatments on SGR, ΔCF, FCR and feed per individual (g) from trial 1 and 2 were each initially described with a second-order (non-linear) polynomial regression of the form: $y = ax^2 + bx + c$. Non-linear polynomial regressions were also used to test the effect of ER on GCOT, as well as being used to calculate U_{opt} (Pettersson and Hedenström, 2000). Exponential regressions were used to analyse the effect of ER on the relationship between MO_2 and swimming speed. Due to the presence of non-normal data, the effect of ER on SGR and ΔCF in both trials was tested with a non-parametric Kruskal–Wallis One-Way analysis of variance (ANOVA) test. When this test identified a significant effect of ER, a Dunn's comparison test was then used to locate a specific *post-hoc* difference in SGR or ΔCF from the control 0 BL s^{-1} ER treatment. After ensuring that data was compliant for normality and homoscedasticity, a repeated measures (RM) Two-Way ANOVA was used to test the effect of swim speed on MO_2 (factor 1) as well as the effect of long-term ER on MO_2 (factor 2). The same Two-Way RM ANOVA was also used to test the effect of the same two factors on GCOT. The optimal (i.e., least cost) swimming speed (U_{opt}) of individual fish was calculated from the non-linear speed-GCOT regression and taken as the speed that yielded a minimum level of GCOT. The effect of long-term ER on U_{opt}, $MO_{2standard}$, MO_{2max}, AMS, and U_{crit} was then tested with individual One-Way ANOVA tests, followed by a Tukey *post-hoc* test for specific pairwise comparisons where appropriate. Significance was accepted at $P \leq 0.05$ and all data are displayed ± standard error. All statistical analyses were performed using SigmaPlot® version 11.0.

RESULTS

EFFECTS OF EXERCISE TRAINING ON JUVENILE HAPUKU GROWTH

Non-linear regressions did not provide convincing evidence that ER was positively linked with weight-specific growth (SGR) for either trial 1 ($F = 1.91$, $R^2 = 0.56$, $P > 0.05$) or trial 2 ($F = 0.56$, $R^2 = 0.27$, $P > 0.05$) (**Figure 1A**). Kruskal–Wallis tests confirmed that ER did not have any effect on the SGR of fish in Trial 2 ($H = 5.23$, $P > 0.05$) where starting weights were higher (**Table 1**) but a strong positive effect of ER on the SGR of fish in trial 1, where starting weights were lower, was identified ($H = 18.93$, $P < 0.01$) (**Figure 1A** and **Table 1**). Specific *post-hoc* comparisons against the control 0.0 BL s^{-1} treatment revealed that

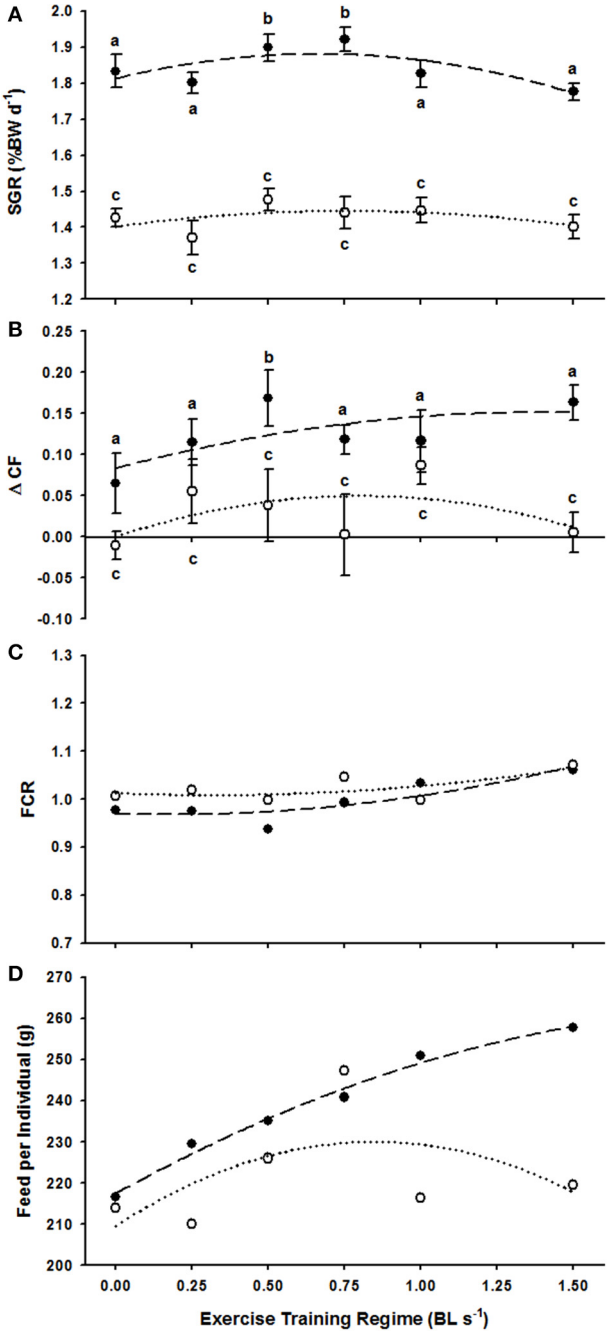

FIGURE 1 | The effect of exercise regimes (ER, in BL s^{-1}) on various production parameters of *P. oxygeneios* in trial 1 (closed circles, broken line) and trial 2 (open circles, dotted line). (A) Average SGR. Regressions are second order non-linear polynomials described as: $y = -0.155x^2 + 0.205x + 1.813$ and $y = -0.075x^2 + 0.114x + 1.402$ for the trial 1 and 2, respectively. **(B)** Average ΔCF. Regressions are: $y = -0.035x^2 + 0.098x + 0.083$ and $y = -0.078x^2 + 0.125x$ for trial 1 and 2, respectively. **(C)** Feed conversion ratio, FCR. Regressions are: $y = 0.058x^2 - 0.02x + 0.97$ and $y = 0.0401x^2 - 0.025x + 1.013$ for trial and 2 fish, respectively. **(D)** Feed intake per individual (g). Regressions are $y = -9.390x^2 + 41.138x + 217.51$ and $y = -28.539x^2 + 48.304x + 209.61$ for the trial 1 and 2 fish, respectively. Dissimilar letters in each of the plots represent a significant difference between swim speed treatments ($P < 0.05$).

Table 1 | The starting weight (g), final weight (g), total feed consumed (g), and number of fish in each of Trial 1 and Trial 2.

Tank speed (BL s^{-1})	Trial 1				Trial 2			
	Start weight (g)	End weight (g)	*N*	Total feed intake (g)	Start weight (g)	End weight (g)	*N*	Total feed intake (g)
0.0	119.9 ± 3.1	341.4 ± 10.1	20	4331.4	170.2 ± 6.1	382.6 ± 12.1	17	3635.2
0.25	131.4 ± 4.7	366.7 ± 9.9	19	4360.1	170.2 ± 8.0	372.8 ± 5.6	17	3359.3
0.5	128.6 ± 4.6	380.6 ± 12.6	20	4702.2	170.9 ± 5.7	397.2 ± 14.1	17	3841.9
0.75	122.7 ± 4.6	365.2 ± 11.8	19	4576.2	187.5 ± 10.6	423.8 ± 19.7	17	4205.7
1.0	133.0 ± 4.3	375.8 ± 7.1	16	4015.2	171.8 ± 10.1	388.4 ± 19.0	17	3678.9
1.5	138.8 ± 3.1	381.9 ± 7.6	17	4384.3	167.4 ± 6.2	372.3 ± 6.2	17	3732.4

All values shown ± standard error.

fish were subject to a significant 3.5% increase in SGR at 0.5 BL s^{-1} ($P < 0.05$) and a 4.8% increase in SGR at 0.75 BL s^{-1} ($P < 0.05$) (**Figure 1A**). No other ER treatment was subject to a change in SGR.

The regressions detailing the link between ER and ΔCF were non-significant within the scale of responses observed in trial 1 ($F = 1.33$, $R^2 = 0.47$, $P > 0.05$) and trial 2 ($F = 0.65$, $R^2 = 0.3$, $P > 0.05$) (**Figure 1B**). ANOVA tests revealed that ΔCF was positively affected by increasing ER in both trial 1 ($H = 12.29$, $P < 0.05$) and trial 2 ($H = 13.76$, $P < 0.05$). However, specific *post-hoc* comparisons against the 0.0 BL s^{-1} control only revealed a significantly higher ΔCF following long-term swimming at 0.5 BL s^{-1} in trial 1 (**Figure 1B**). Therefore, in addition to the positive effect on SGR, fish at 0.5 BL s^{-1} had a relatively deeper body shape.

FCR varied little as a function of ER across trial 1 ($F = 4.98$, $R^2 = 0.77$, $P > 0.05$) and trial 2 ($F = 1.67$, $R^2 = 0.53$, $P > 0.05$) (**Figure 1C**). Feed intake per individual (g) was positively related to ER in trial 1 fish ($F = 115.48$, $R^2 = 0.98$, $P < 0.05$) but showed no relationship with ER in trial 2 ($F = 0.78$, $R^2 = 0.34$, $P > 0.05$, **Figure 1D**).

EFFECTS OF EXERCISE TRAINING ON THE SWIMMING PERFORMANCE OF JUVENILE HAPUKU

MO_2 increased linearly with swimming speed for each of the 0.0, 0.75, and 1.5 BL s^{-1} ER groups (linear regressions with $R^2 = 0.78$, $R^2 = 0.78$, $R^2 = 0.76$, and $P < 0.05$ for the 0.0, 0.75, and 1.5 BL s^{-1} ER groups, respectively, **Figure 2A**) and a highly significant effect of swimming speed on MO_2 was confirmed from the Two-Way RM ANOVA tests ($F = 136.11$, $P < 0.01$). There was, however, no significant difference in MO_2 between the three ER treatments ($F = 1.41$, $P > 0.05$) and there was no significant interaction between swimming speed and ER on MO_2 ($F = 0.61$, $P > 0.05$).

GCOT showed a significant parabolic relationship with swimming speed for each of the 0.0, 0.75, and 1.5 BL s^{-1} ER groups ($R^2 = 0.74$, $R^2 = 0.73$, $R^2 = 0.73$, and $P < 0.05$ for the 0.0, 0.75, and 1.5 BL s^{-1} ER groups, respectively, **Figure 2B**) and a highly significant effect of swimming speed on GCOT was once again confirmed with the Two-Way RM tests ($F = 138.47$, $P < 0.01$). However, there was no significant difference in GCOT between the three ER groups ($F = 0.51$, $P > 0.05$) and there was no interactive effect of swimming speed and ER on GCOT ($F = 0.46$, $P > 0.05$). U_{opt} estimations were also not significantly different between the three ER treatments ($F = 1.26$, $P > 0.05$) and were essentially identical to the pooled U_{opt} estimation of 1.86 BL s^{-1} with a GCOT minima of 0.03 mg O_2 kg^{-1} BL^{-1}.

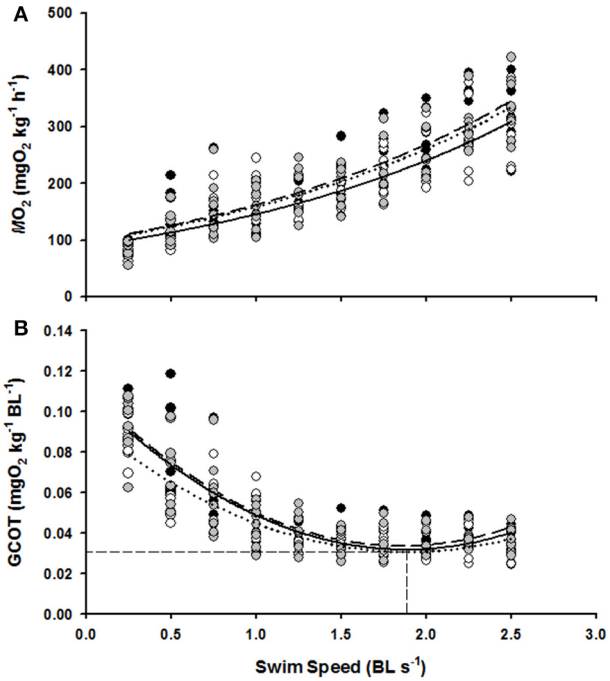

FIGURE 2 | The effect of swim flume speed (BL s^{-1}) on the MO_2 (A) and GCOT (B) of juvenile hapuku raised for 6 weeks at either 0.0 BL s^{-1} (black circles, broken line), 0.75 BL s^{-1} (open circles, dotted line), or 1.5 BL s^{-1} (gray circles, solid line). MO_2 regressions are $y = 90.38e^{0.5438x}$, $y = 79.47e^{0.5531x}$, and $y = 89.26e^{0.5399x}$, respectively. GCOT regressions are $y = 0.023x^2 - 0.084x + 0.111$, $y = 0.018x^2 - 0.067x + 0.094$, and $y = 0.021x^2 - 0.081x + 0.109$, respectively. As no significant differences were detected between ER treatments in GCOT (see Results), the horizontal dashed lines refers to the calculated pooled $GCOT_{min}$ (0.03 mg O_2 kg^{-1} BL^{-1}) and the vertical dashed line refers to pooled U_{opt} (1.86 BL s^{-1}).

Long-term exposure to the three ER treatments had no significant effect on $MO_{2standard}$ ($F = 1.17$, $P > 0.05$), MO_{2max} ($F = 1.15$, $P > 0.05$), AMS ($F = 0.75$, $P > 0.05$), or U_{crit} ($F = 2.63$, $P > 0.05$) (**Table 2**).

Table 2 | The average weight (g), standard metabolic rate ($MO_{2standard}$), maximum metabolic rate (MO_{2max}), and aerobic metabolic scope (AMS) of juvenile hapuku (measured as mg O_2 kg^{-1} h^{-1}) as well the critical swimming speed (U_{crit}, BL s^{-1}) of juvenile hapuku raised for 6 weeks at either 0.0, 0.75, or 1.5 BL s^{-1} and measured in a swim-flume respirometer.

	Swim speed treatment		
	0.0 BL s^{-1}	**0.75 BL s^{-1}**	**1.5 BL s^{-1}**
Average weight (g)	469.12 ± 5.96	496.38 ± 8.14	481.44 ± 6.77
Standard metabolic rate ($MO_{2standard}$, mg O_2 kg^{-1} h^{-1})	91.33 ± 5.37	80.58 ± 5.46	90.67 ± 5.80
Maximum metabolic rate (MO_{2max}, mg O_2 kg^{-1} h^{-1})	324.72 ± 7.44	294.05 ± 5.99	337.90 ± 8.59
Aerobic metabolic scope (AMS, mg O_2 kg^{-1} h^{-1})	233.39 ± 9.04	213.47 ± 5.49	247.23 ± 8.23
Critical swimming speed (U_{crit}, BL s^{-1})	2.72 ± 0.13	2.55 ± 0.08	2.94 ± 0.14

No significant effect of swimming speed treatment was detected in any of the listed variables ($P > 0.05$).
All values shown ± standard error.

RECONCILING THE COST OF SWIMMING AND GROWTH

A summary of hapuku metabolic costs across a temperature range of 15–24°C was amalgamated and graphically represented (**Figure 3**) for the purpose of reconciling metabolic components against available AMS at 17°C. $MO_{2standard}$ values for 15°C and 21°C were measured in a different study in similarly sized fish using a static respirometry system (Khan et al., in press) and the line between these two values intersected 17°C at 91.31 mg O_2 kg^{-1} h^{-1} which is very similar to the $MO_{2standard}$ estimate from the current study (87.53 ± 5.21 mg O_2 kg^{-1} h^{-1}) at 17°C (**Table 2**). SDA estimates were also measured in the same previous study for fish fed a 1.5% BW d^{-1} ration at both 15 and 21°C (Khan et al., in press). An estimate of SDA at 17°C was then interpolated from the straight line function between these two SDA values ($Q_{10} = 3.44$, $MO_2 = 25.45temp - 242.95$). It was therefore assumed that peak SDA follows a linear relationship between these two temperatures when fed the same-sized ration. (NB. ration size varied 1.3–1.8% BW d^{-1} in the current study so was close to the standard 1.5% BW ration in Khan et al., in press). ER had no significant effect on swimming costs at 17°C (see above) so the MO_2 values from each ER were pooled to calculate an average cost of swimming at 0.25, 0.5, 0.75, 1.0, and 1.5 BL s^{-1}. MO_{2max} values are shown as the highest and lowest estimates from the current study (i.e., 1.5 BL s^{-1} = 337.90 ± 8.59 mg O_2 kg^{-1} h^{-1} and 0.75 BL s^{-1} = 294.05 ± 5.99 mg O_2 kg^{-1} h^{-1}, respectively, **Table 2**).

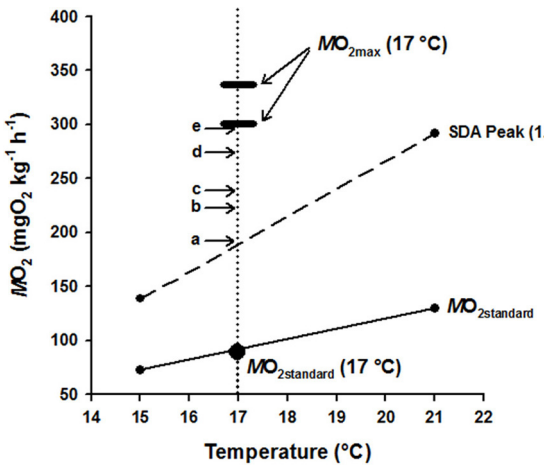

FIGURE 3 | Graphical summary of hapuku metabolic components as a function of temperature (15–21°C) with particular detail at 17°C, allowing the costs of specific dynamic action (SDA) and swimming to be balanced within the boundaries of aerobic metabolic scope (AMS = MO_{2max} - $MO_{2standard}$). $MO_{2standard}$ at and between 15 and 21°C (small filled circles adjoined by the lowest solid line) was taken from Khan et al. (in press) using a static respirometry system whilst $MO_{2standard}$ at 17°C (large filled circle) was measured directly within the current study using the swim-flume respirometer. The two small filled circles adjoined by the broken line show the peak in the SDA response of 300–500 g hapuku fed a 1.5% BW d^{-1} ration at and between 15 and 21°C (Khan et al., in press). It is likely that the SDA costs measured at 15 and 21°C can be used to accurately interpolate the feeding costs of fish in the current study at 17°C because the fixed 1.5% BW d^{-1} ration of Khan et al. (in press) closely approximates the 1.3–1.8% BW d^{-1} *ad libitum* ration level of fish from the different ER treatments. The thickened horizontal dash indicates the MO_{2max} of 300–500 g hapuku measured in the swim flume at 17°C during the current study. Overlaid above $MO_{2standard}$ (solid line) and peak SDA (broken line) are horizontal arrows with letters (a–e) showing the additional measured costs of swimming from the current study at 17°C as follows: (a) 0.25 BL s^{-1}, (b) 0.5 BL s^{-1}, (c) 0.75 BL s^{-1}, (d) 1.0 BL s^{-1}, and (e) 1.5 BL s^{-1}. The vertical dotted line therefore represents the transect through the accumulated costs of maintenance ($MO_{2standard}$), feeding/growth (SDA) and exercise of *P. oxygeneios* at 17°C and shows that the costs of SDA and exercise fall comfortably within the limits of available aerobic metabolic scope at this temperature (see Results and Discussion for more detail).

DISCUSSION

In order to validate the model of Davison and Herbert (2013), exercise-induced growth in juvenile hapuku would be expected in the range of ~0.4–0.5 BL s^{-1}. However, the current does not provide compelling evidence of exercise-induced growth at 17°C (**Figure 1**) which is a stark contrast to salmonids that reportedly show a ≤40% increase in growth from sustained exercise in the region of 0.75–1.5 BL s^{-1} (Davison and Goldspink, 1977; Houlihan and Laurent, 1987; Jørgensen and Jobling, 1993). Indeed, hapuku with an average starting weight of 130 g in Trial 1 only showed a maximum of a 4.8% increase in growth at 0.5 and 0.75 BL s^{-1}, respectively, (**Figure 1A**) whereas the larger 170 g (starting weight) hapuku showed no sign of exercise-induced growth in Trial 2 (**Table 1**). Earlier studies on salmonids considered that the ER at which optimal growth is ascertained (i.e., $ER_{opt\ growth}$) was attributed to their active ecotype, as well as the physiological and behavioral requirements of schooling, migration and river spawning [e.g., position holding in strong water flows (Jobling et al., 1993)]. Hapuku would not be described as highly active so the data is consistent with

the view of Jobling et al. (1993). However, the recent review on exercise-induced growth in fish by Davison and Herbert (2013) went further to propose that ER $_{opt\ growth}$ is a function of AMS. Most salmonids, with their active ecotype and high AMS (∼350–500 mg O_2 kg^{-1} h^{-1}), show exercise-induced growth at relatively fast swimming speeds (Walker and Emerson, 1978; Houlihan and Laurent, 1987; Jørgensen and Jobling, 1993; Bugeon et al., 2003) and therefore provide data to support the upper end of the Davison and Herbert (2013) model. In contrast, the lower end of the model is based on species such as gadoids that have a small AMS in the region of ∼150–200 mg O_2 kg^{-1} h^{-1} (Hammer, 1994; Karlsen et al., 2006) and show little to no growth response to exercise-training (Bjørnevik et al., 2003; Karlsen et al., 2006). On the basis of these observations, the current study aimed to assess the AMS – ER $_{opt\ growth}$ model of Davison and Herbert (2013) by testing whether the ∼300 mg O_2 kg^{-1} h^{-1} AMS level of Khan et al. (2014) does indeed lead to an ER $_{opt\ growth}$ of 0.4–0.5 BL s^{-1}. At least for trial 1, the Davison and Herbert (2013) model prediction does appear to provide a reasonable fit. However, the lack of exercise-induced growth in trial 2 is not consistent with the Davison and Herbert (2013) model and the very modest levels of growth acceleration do not validate the model for this novel species.

The AMS values measured from 480 g hapuku at the end of the current study ranged from 213 to 247 mg O_2 kg^{-1} h^{-1} (**Table 2**) and are therefore lower than the 300 mg O_2 kg^{-1} h^{-1} AMS value ascertained for 180 g hapuku at 17°C in the study of Khan et al. (2014). Whilst these larger AMS values were used initially to formulate our hypothesis, the recently established values of AMS are considered more valid because they originate from a size class of fish that corresponds to the current ER $_{opt\ growth}$ data. However, applying these lowered AMS values to the model of Davison and Herbert (2013) predicts an ER $_{opt\ growth}$ of between 0.15 and 0.3 BL s^{-1} which does not correspond to the observed ER $_{opt\ growth}$ range of fish in trial 1, or even the total lack of exercise-induced growth in trial 2 (**Figure 1A**). These data further suggest that the relationship between AMS and ER $_{opt\ growth}$ is not validated in this species.

In relation to the second model of Davison and Herbert (2013), the ER $_{opt\ growth}$ range observed in trial 1 (0.5–0.75 BL s^{-1}, **Figure 1**) does not even vaguely correspond to the measures of U_{opt} in the current study (1.86 BL s^{-1}, **Figure 2B**). The U_{opt} estimation for juvenile hapuku was unaffected by ER and is considerably higher than one might expect for a species that is less active than Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, and brook charr *Salvelinus fontinalis* which all have U_{opt} values in the range of 0.9–1.1 BL s^{-1} (Beaumont et al., 2000; Deitch et al., 2006; Tudorache et al., 2011). Atlantic cod and gilthead seabream *Sparus aurata* also have unusually high U_{opt} estimations [ranging from 1.2 to 1.6 BL s^{-1} in the cod and up to 2.3 BL s^{-1} in the gilthead seabream (Schurmann and Steffensen, 1997; Steinhausen et al., 2010)]. Alternative methods of calculating the minimum COT (i.e., those suggested by Pettersson and Hedenström, 2000) produce a similarly high U_{opt} estimate of 1.84 BL s^{-1} for the pooled GCOT data (**Figure 2B**). It may be that these less active ecotypes do not have an ecologically functional or relevant U_{opt} as would be the case for migratory or highly active species (Hinch and Rand, 2000; Tudorache et al., 2011) though this is speculation and requires further investigation.

The hypothesis that AMS places a capacity limitation on exercise-induced growth (Davison and Herbert, 2013) is not supported by the current data for juvenile hapuku. For 480 g hapuku at 17°C, the costs of exercise and SDA [which can be comprised of up to 80% protein synthesis (Coulson and Hernandez, 1979; Brown and Cameron, 1991; Seth et al., 2010; Li et al., 2013)] are easily accommodated within available AMS, even at the highest swimming speed used in the growth trials (1.5 BL s^{-1}, **Figure 3**). It is generally accepted that the energetic costs associated with SDA are largely comprised of post-absorptive protein synthesis and is thought to represent the cost of growth (Whiteley et al., 2001; Grigoriou and Richardson, 2008; Secor, 2009) and, in less active species with low AMS, SDA often consumes a large proportion of AMS potential (Jobling, 1983; Soofiani and Priede, 1985; Jordan and Steffensen, 2007). This has led researchers to propose that an inability to reconcile the metabolic costs of growth and exercise simultaneously would either lead to a reduction in the rate of protein synthesis (as a prioritization of exercise over growth, Davison and Herbert, 2013) or, as predicted for the European eel *Anguilla anguilla* in the study of Owen (2001), a reduction in swimming activity as a prioritization of growth over exercise. Therefore, with an ability to accommodate the costs of exercise and growth simultaneously and with metabolic costs of swimming (**Figure 2**) and SDA not vastly different to other ecotypes (Fu et al., 2005; Jordan and Steffensen, 2007; Ohlberger et al., 2007; Yanase et al., 2012; Frisk et al., 2013), it is proposed that the weak exercise growth response of hapuku is a species-specific effect and not due to capacity limitation of aerobic metabolism.

The data in **Figure 3** provides evidence that AMS does not limit the ability of juvenile hapuku to swim and grow simultaneously but, on a cautionary note, it does not take into the account the extra metabolic costs of spontaneous activity (Boisclair and Tang, 1993; Tang et al., 2000) nor does it necessarily prove that hapuku have the metabolic capacity to grow *faster* whilst swimming. With respect to the latter point, the SDA costs of supplementary fast growth from exercise were not measured within static respirometry chambers (Khan et al., in press) and is therefore still not yet resolved. Interestingly, there is other recent data suggesting that the costs of SDA and exercise can act additively in the darkbarbel catfish *Peltebargus vachelli* (Li et al., 2010) and the sea bass *Dicentrarchus labrax* (Altimiras et al., 2008) to the point where total costs exceed measured MO_{2max}. This is relevant to the current discussion as it suggests a potential disconnect between AMS and the combined costs of exercise and growth. More importantly, this data opposes the AMS - ER $_{opt\ growth}$ hypothesis of Davison and Herbert (2013) as exercise and growth could potentially occur simultaneously in catfish and sea bass without their costs being limited by AMS. The presence of additive SDA has not yet been addressed in hapuku and, whilst this species appears suited to U_{crit} swimming tests in a swim-flume respirometer, feeding attempts have not yet been successful. To investigate this issue further, it may be necessary to implement a gavage protocol or directly infuse food or amino acids into the gut or bloodstream (Brown and Cameron, 1991; Li et al., 2010).

CONCLUSION

The data from the current study is not consistent with the hypothesis of Davison and Herbert (2013) that AMS sets a limit to, and therefore determines, the likelihood of seeing exercise-induced growth in finfish aquaculture species such as hapuku. This was essentially based on the fact that (i) juvenile hapuku showed a modest and inconsistent exercise-induced growth response in a narrow band of swimming speeds (0.5–0.75 BL s^{-1}), and (ii) the AMS of these fish appears sufficient to accommodate the physiological costs SDA and swimming simultaneously. It may be that this species is generally not responsive to exercise training but, before that conclusion is reached, future research should possibly strive to examine the response of different-sized hapuku across a greater range of (optimal) temperatures as a means of disentangling the potential role of these factors in exercise-induced growth (e.g., Brown et al., 2011).

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Comparison of swimming capacity and energetics of migratory European eel (*Anguilla anguilla*) and New Zealand short-finned eel (*A. australis*)

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The spawning migration of the European eel (*Anguilla anguilla*) can cover more than 6000 km, while that of the New Zealand short-finned eel (*A. australis*) is assumed to be approximately 3000 km. Since these species are expected to show adaptive traits to such an important lifetime event, we hypothesized differences in swimming capacity and energetics as a response to this adaptation. In an experimental swimming respirometer set-up, critical swimming speed (U_{crit}), optimal swimming speed (U_{opt}), mass specific oxygen consumption rate ($\dot{M}O_2$), standard metabolic rate (SMR), active metabolic rate at U_{crit} (AMR_{crit}) and at U_{opt} (AMR_{opt}), the minimum cost of transport at U_{opt} (COT_{min}), and the scope for activity, were assessed and compared between the species. With a similar body length and mass, European eels showed ca. 25% higher values for both U_{crit} and U_{opt}, and 23% lower values for COT_{min}, compared to New Zealand short-finned eels. However, SMR, AMR_{crit}, AMR_{opt}, and scope for activity did not differ between the species, indicating very similar swimming physiology traits. This study discusses physiological aspects of long distance migration and provides recommendations for (a) swimming respirometry in anguilliform fish, and (b) telemetry research using externally attached pop-up tags.

Keywords: ecology, physiology, respirometry, biometry, fish

Introduction

Fish species have evolved strategies, which enable them to use different types of habitats during their life cycle (for reviews see Lucas and Baras, 2001; Tesch, 2003). Facultative catadromic species such as anguillid eels (Tesch, 2003; Aoyama, 2009; Jellyman et al., 2009) use marine habitats for reproduction and freshwater or brackish habitats for growth and differentiation. During an extremely complex life cycle, anguillid eels develop through oceanic larval (leptocephali) and juvenile stages (glass eels), and many years of growth and differentiation in freshwater or brackish habitats, into a migratory "silver" stage. At the end of their lives, these silver eels perform an oceanic reproductive migration to their spawning grounds where they spawn and subsequently die (semelparity; Tesch, 2003).

The location of the spawning site of the European eel (*Anguilla anguilla*) is still a mystery but early studies strongly indicate the Southwest Sargasso Sea based on the location of the smallest larvae found (Schmidt, 1923, 1925). Since the start of the spawning migration occurs in autumn and the first glass eels arrive at European coasts in spring it is suggested that migrating silver eels cover 6000 km or more within 6 months to reach their spawning grounds in April (Tesch, 2003). The newly hatched leptocephalus larvae subsequently drift with the golf stream for a year until they are recruited as glass eels at the European coast during the next spring (Schmidt, 1923; Tesch, 2003). However, no adult spawning eels have been observed in the Sargasso Sea to date, nor have eggs been found.

Equally mysterious is the spawning migration of the New Zealand short-finned eel (*A. australis*). Only a few leptocephalus larvae have been found in an area located Northwest of the Fiji Islands (Castle, 1963). Based on these findings, Castle (1963) suggests a potential spawning site between the Fiji Islands and Tahiti (170°W by 18°S), while Jellyman (1987) argues that a more Northern area (between 150 and 170°W by 5 and 15°S) was likely, based on literature data of oceanography, body size, time of larval recruitment, otolith microstructure, and gonadal development for migrating silver eels. The linear migration distance from the most distant area of distribution, i.e., Southern Victoria and Tasmania (Dijkstra and Jellyman, 1999) to these potential spawning sites would amount to ca. 3000 km.

Assuming similar migration behavior and strategies for both species during spawning migration, but greatly different distances, the total energy expenditure would be higher for European than for New Zealand short-finned eels. Therefore, we hypothesize that swimming capacity and energetics correspond with migration distances, resulting in an overall higher swimming capacity and a lower energy expenditure over distance swum for European than for New Zealand short-finned eels.

TABLE 1 | Data on biometrics (body length, mass, maximum cross sectional area), swimming capacity (critical swimming speed, U_{crit}), and swimming energetics of European (EU) and New Zealand short-finned eels (NZ).

	EU	NZ	*p*-value
Body length (mm)	773 ± 37	746 ± 25	0.56
Body mass (g)	905.67 ± 145.29(a)	808.69 ± 79.09(a)	0.57
Maximum cross sectional area (mm^2)	167 ± 4	173 ± 4	0.31
SMR($mgO_2kg^{-1}h^{-1}$)	45.65 ± 2.12	50.52 ± 3.11	0.22
U_{crit}($m\ s^{-1}$)	0.94 ± 0.02	0.74 ± 0.03	**0.0002**
U_{opt}($m\ s^{-1}$)	0.64 ± 0.03	0.51 ± 0.02	**0.004**
COT_{min}($mgO_2kg^{-1}\ m^{-1}$)	54.48 ± 2.13	67.91 ± 2.17	**0.0008**
AMR_{opt}($mgO_2kg^{-1}h^{-1}$)	125.55 ± 5.76	124.95 ± 4.5	0.94
AMR_{crit}($mgO_2kg^{-1}h^{-1}$)	206.44 ± 11.41	197.26 ± 9.49	0.55
Scope for activity ($mgO_2kg^{-1}h^{-1}$)	160.25 ± 11.27	151.29 ± 9.58	0.56

*Oxygen consumption rate ($\dot{M}O_2$, $mgO_2kg^{-1}\ h^{-1}$, see **Figure 1**) was expressed as a exponential function of swimming speed (U, $m\ s^{-1}$): $\dot{M}O_2 = SMRe^{cU}$, with SMR the standard metabolic rate, e Euler's constant, and c constant. From this function, optimal swimming speed (U_{opt}, $m\ s^{-1}$), minimum Cost of Transport (COT_{min}, $mgO_2\ kg^{-1}\ km^{-1}$), active metabolic rate at U_{opt} (AMR_{opt}, $mgO_2kg^{-1}\ h^{-1}$), and at U_{crit}, the critical swimming speed (AMR_{crit}) and the scope for activity ($mgO_2kg^{-1}h^{-1}$) were derived. Values are mean ± SE, significance was accepted at $p < 0.05$ (bold), t-test, $N = 7$.*

Materials and Methods

Wild female silver European eels (*A. anguilla*, $N = 7$; body mass and body length, see **Table 1**; silver index 4–5, Durif et al., 2005) were caught by means of fykes in Lake Veerse, The Netherlands, at the end of October 2011, and transported to our laboratory in Leiden in large barrels with a small amount of water.

Wild female silver New Zealand short-finned eels (*A. australis*, $N = 7$; body mass and body length, see **Table 1**, migratory pre-reproductive stage, Lokman et al., 1998) were caught by means of fykes in Lake Ellesmere, Christchurch, New Zealand, in March 2011, and transported to The Netherlands in aerated plastic bags with a small amount of water, fitted into cooled polystyrene boxes (5–10°C), with the journey lasting for 2.5 days.

After arrival in the laboratory in Leiden, The Netherlands, eels were acclimated for ca. 2 weeks in a 4000 L recirculation system, supplied with natural seawater (28 ± 1 ppt) at 18 ± 1°C (water and air temperature) with an air saturation of 75–85%, situated in a climate cell for constant conditions. Fish were kept under dimmed light to reduce stress before and during the trials. Migratory eels cease feeding, so they were not fed during the entire period of time. Since both species originated from brackish water bodies, and (pre) migrating eels are very adaptive to rapid changes in salinity (Tesch, 2003), adaptation to the higher salinity of the holding facility was not assumed to be stressful. Indeed, all eels responded very well to transport and transition to seawater, without symptoms of discomfort or stress, and the animals appeared lively and agile. The eels kept their silver stage during the entire experimental period.

Preparation and Acclimation

For preparatory handling before swimming trials i.e., measurement of body mass, length, height, and width for calculation of cross sectional areas, eels were anesthetized with clove oil, dissolved in 96% ethanol at a ratio of 1:10, which was dosed 1 ml in 1 l seawater. All eels were completely unresponsive under anesthesia when measured and weighed. Biometric data show no significant differences between species in body length or body mass (**Table 1**).

Subsequently, the eels were transferred to 14 identical 127 l Blazka-type swimming tunnel (swim section length: 1150 mm, diameter: 190 mm, identical flow profiles; described in van den Thillart et al., 2004) connected to the recirculation system, in which the swimming trials (seven European and seven New Zealand short finned eels) were performed simultaneously in order to avoid time related effects.

Upon transfer to the swimming tunnels, the eels recovered after 1–5 min, showing routine activity. The animals were allowed to fully recover over a period of 16–24 h, which is considered sufficient for swimming fish (e.g., Lee et al., 2003; Svendsen et al., 2010). The water speed was set at 0.1 m s^{-1}, at which they would remain coiled up against the rear grid of the swimming tunnels.

This low velocity ensured an equal distribution of oxygenated water in the tunnel (Burgerhout et al., 2011; Methling et al., 2011; Tudorache et al., 2014). The tunnels were covered with a black plastic sheet in order to reduce stress due to visual disturbance.

Swimming Trials

Swimming capacity was estimated by critical swimming speed (U_{crit}), the maximum sustained swimming speed (Brett, 1964), and mass specific oxygen consumption rate ($\dot{M}O_2$) and derived energetic values provided information about energy use during long term migration.

At water speeds of a minimum of 0.4 m s^{-1} for European and 0.3 m s^{-1} for New Zealand short finned eels, fish would orientate themselves against the stream and hold position in the tunnel using a regular swimming mode, characterized by a steady anterior position, visually uniform tail beat frequency and amplitude. Oxygen consumption rate data below these swimming velocities were not included in the analysis.

First, the animals were subjected to a U_{crit}-test. Water velocity was increased in increments of 0.1 m s^{-1} at intervals of 20 min (Methling et al., 2011) until the fish fatigued, i.e., refused to swim and was flushed against the downstream grid of the tunnel where it remained for at least 20 s. After fatigue, fish were allowed to recover at a water speed of 0.1 m s^{-1} for 16–24 h.

Subsequently, for the calculation of $\dot{M}O_2$, eels were subjected to a series of swimming speeds ranging from 0.3 to 0.9 m s^{-1} with 0.1 m s^{-1} increments and 60 min intervals. Flushing with oxygenated water from the recirculation system occurred during the first 30 min (air saturation 82.7 ± 5.3%), and oxygen concentration ($[O_2]$) was measured during the last 30 min of each swimming period, to allow a steady measurement without the possible effects of a fish stressed by previous changes in swimming velocity. $[O_2]$ was measured with a galvanic oxygen electrode (type Inpro 6415, Mettler Toledo, The Netherlands) and logged with a HP 34970A multichannel logger and controller, connected to two 40-channel multiplexers (34907 and 34901 A). Data were sampled at a rate of 0.1 Hz. The air saturation never fell below 70% during the test and water temperature was constantly 18 ± 1°C. Background oxygen consumption rate of the system was previously measured (van den Thillart et al., 2004) and <2% h^{-1}. After trials, eels were removed and reused in other studies.

Data Analysis

U_{crit} was calculated according to the equation:

$$U_{crit} = U_i + [\Delta U\,(T_i\,\Delta T^{-1})],$$

where U_i is the highest velocity maintained for the entire 20 min interval, ΔU is the velocity increment (0.1 m s^{-1}), T_i is the duration of the final (fatigue) step and ΔT is the time interval (20 min; Brett, 1964).

$\dot{M}O_2$ ($mgO_2kg^{-1}\ h^{-1}$) was fitted as a function of swimming speed (U) to the exponential equation:

$$\dot{M}O_2 = SMR\,e^{cU},$$

with SMR, the standard metabolic rate; e, Euler's constant; and c, being constant. The optimal swimming speed, (U_{opt}), i.e., the swimming speed with minimum energy consumption, was calculated from this exponential function by

$$U_{opt} = 1/c$$

and the minimum cost of transport (COT_{min} in $mgO_2kg^{-1}\ km^{-1}$), i.e., the lowest cost over distance, swum at U_{opt}, was calculated by

$$COT_{min} = \frac{\dot{M}O_2\,(U_{opt})}{U_{opt}}$$

(Petterson and Hedenström, 2000).

Resulting swimming speeds (U_{crit}, U_{opt}) and other calculated parameters were corrected for the solid blocking effect according to Bell and Terhune (1970):

$$U_F = U_T\,(1 + \varepsilon_S)$$

with U_F the corrected speed, U_T the original speed, and ε_S the fractional error quotient:

$$\varepsilon_S = \tau\lambda(A_O/A_T)^{3/2}$$

with τ a dimensionless factor depending on flume cross-sectional shape, λ a shape factor for the test object (0.5), A_O the maximum cross-sectional area of the fish, and A_T the cross-sectional area of swimming section (Bell and Terhune, 1970).

The critical (AMR_{crit}) and optimal active metabolic rate (AMR_{opt}) are the $\dot{M}O_2$ at U_{crit} and at U_{opt}, respectively. The scope for activity is the difference between SMR and AMR_{crit}.

Statistics

Data (U_{crit}, U_{opt}, body length, body mass, maximum cross sectional area, SMR, COT_{min}, AMR_{opt}, AMR_{crit}, Scope for activity, $\dot{M}O_2$) and residuals were tested for normal distribution with a Kolmogorov-Smirnoff test; after confirmation ($p < 0.05$, $N = 7$), they were compared between eel species using a student-*t*-test (SigmaPlot v. 11, Systat systems inc. USA). In order to test for an effect of the variation of body mass on $\dot{M}O_2$, and body length on U_{crit} and U_{opt}, respectively, an ANCOVA was performed to compare absolute values of metabolic rate with mass and size as corresponding covariates in the analyses and to account for differences in mass and size among individuals and between species. Significance was determined in all cases at $p < 0.05$. Data are given as mean ± SE.

Ethics Statement

This study complied with the Dutch Law on Animal Experiments and was approved by the Animal Ethical Committee of Leiden University (DEC# 10231). All measurement was performed under clove oil anesthesia, and all efforts were made to minimize suffering and reduce the number of animals used.

Results

After correction for the solid blocking effect, critical swimming speed (U_{crit}, **Table 1**) was ca. 25% higher in European than

in New Zealand short-finned eels ($p < 0.01$), as was optimal swimming speed (U_{opt}; $p < 0.01$, **Table 1**). Mass specific oxygen consumption rates ($\dot{M}O_2$; **Figure 1**) were lower in European than in New Zealand short-finned eels at all speeds above 0.5 m s^{-1} ($p < 0.01$; **Figure 1**), as were minimum cost of transport (COT_{min}) values ($p < 0.01$, **Table 1**). However, other energetic values (**Table 1**) such as the extrapolated standard metabolic rate (SMR), the active metabolic rate at U_{opt} (AMR_{opt}) and at U_{crit} (AMR_{crit}), and the scope for activity, were not significantly different between species ($p > 0.05$). ANCOVA analysis confirmed that differences in absolute oxygen consumption rates and swimming velocities (U_{crit} and U_{opt}) did not depend on differences in body mass between the species (*p*-values for EU and NZ, respectively: $\dot{M}O_2$ at 0.5 m s^{-1} 0.13 and 0.27; $\dot{M}O_2$ at 0.6 m s^{-1} 0.14 and 0.23; U_{crit} 0.21 and 0.32; U_{opt} 0.13 and 0.22).

Discussion

To our knowledge, this is the first direct estimate of swimming capacity and energetics of New Zealand short-finned eel (*A. australis*) and its comparison with European eel (*A. anguilla*). The distance of their spawning migrations differs greatly, with 6000 and 3000 km, respectively. Based on a possible evolutionary adaptation to strongly different migration distances between the species, we hypothesized differences in swimming capacity and energetic parameters. Indeed, the results show that regardless the similarity in size and mass of the tested animals, the critical (U_{crit}) and the optimal swimming speed (U_{opt}) were significantly higher in European than in New Zealand short-finned eels, indicating an increased swimming capacity. However, when extrapolating U_{crit} or U_{opt} to oxygen consumption rate ($\dot{M}O_2$), the resulting critical or optimal active metabolic rates (AMR_{crit}, AMR_{opt}) were similar between the species. These results suggest no difference in maximum and minimum aerobic expenditure rates and therefore similar energetic profiles between the species.

The observed differences in swimming capacity (U_{crit} and U_{opt}), contrasting the similar energetic profiles (AMR_{crit} and AMR_{opt}), may be explained in terms of muscle power output. Altringham and Ellerby (1999) showed that muscle function can vary among species. For example, it appears that many species have different distributions of slow, aerobic muscles, with a decrease of muscle twitch speed from anterior to posterior along the body axis (Altringham et al., 1993; Davies and Johnston, 1993; Rome et al., 1993; Davies et al., 1995; Altringham and Block, 1997). However, Ellerby et al. (2001) showed that the change in isometric properties of slow muscles along axial location is less marked in eels than in most other species. Time from stimulus to peak force does not change significantly with axial position (Ellerby et al., 2001). Since anguilliform swimmers show uniform muscle kinetics along the body axis, swimming with different tail beat amplitude or frequency, and therefore stride length (the distance covered per tail beat cycle; Videler and Wardle, 1991), can possibly account for the differences in swimming capacity observed between European and New Zealand eels. Future research should clarify if swimming kinematics is accountable for the observed differences in swimming capacity and energetics as related to red muscle power output.

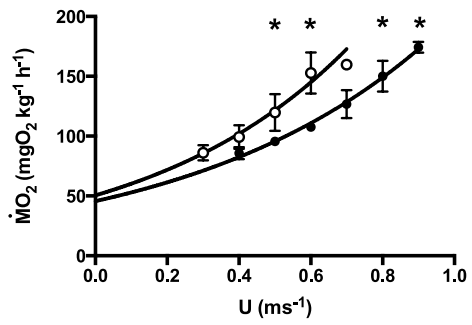

FIGURE 1 | Relative oxygen consumption rate ($\dot{M}O_2$, mgO_2 kg^{-1} h^{-1}) as a function of swimming speed (U, m s^{-1}) for EU (full circles) and NZ (empty circles). Exponential function $MO_2 = SMRe^{cU}$, with SMR the standard metabolic rate, e Euler's constant and c constant, and U swimming speed (for values see **Table 1**). Data are mean ± SE, $r^2 = 8.9 \pm 2.2$. Curve fitting data are given in **Table 1**. *indicates significant difference between species at the respective speed: *t*-test, $p < 0.05$, $N = 7$.

Similar to AMR_{crit} and AMR_{opt}, the standard metabolic rates (SMR) of European and New Zealand short finned-eels do not differ. Since the SMR is defined as the metabolic rate of a resting, fasting fish at a particular temperature (Videler, 1993), it is the sum of all energetic processes occurring during rest, including the response to stress (Sloman et al., 2000). Cortisol is the end product of the Hypothalamic-Pituitary-Interrenal axis in fish, which is activated as a physiological response to stress (Wendelaar-Bonga, 1997). Blood cortisol levels regulate the basic metabolic rate through various processes, including heart beat rates (Davis and Schreck, 1997). Therefore, similar SMRs indicate similarity in stress levels as responses to handling, housing, and transport. Also, it has been shown that swimming exercise suppresses stress and cortisol based stress effects in fish (Milligan, 1996). It therefore can be assumed that observed differences in swimming speeds may not be based on different stress levels or maintenance costs between European and New Zealand short-finned eels. However, a definitive conclusion can only be made by simultaneously measuring blood cortisol levels.

Regardless the COT_{min} or swimming speeds during migration, if the spawning migration can be completed, reproduction successfully accomplished and enough fat incorporated into the eggs for the larvae to survive until they can independently feed, is not to be predicted from the present data for both, European and New Zealand short finned-eels. Additional research will have to be conducted.

Implications for Telemetry Studies

The optimal swimming speed (U_{opt}) for European eels, i.e., the velocity with the lowest cost of transport (COT_{min}) and therefore the presumed migration speed, is similar to previous findings (Palstra et al., 2008; Burgerhout et al., 2011; Methling et al., 2011). According to Tesch (2003), European eels would require a migration speed of 35 km d^{-1} or 0.4 m s^{-1} to reach the postulated spawning ground in the Sargasso Sea within the expected 6 months. Field studies (e.g., Aarestrup et al., 2009), show that eels equipped with an external satellite tag

travel at an average migration speeds of 5–25 km d^{-1}, i.e., 0.1–0.3 m s^{-1}, significantly lower than the required migration or measured optimal swimming speed. However, the suggestion that these eels are impaired by the added drag of the tag and consequently reach lower migration speeds can be dismissed. Various recent experimental studies on the swimming capacity of externally tagged eels (Burgerhout et al., 2011; Methling et al., 2011; Tudorache et al., 2014) showed that U_{opt}-values of tagged eels were similar to those of untagged individuals, suggesting that other factors may compensate for the added drag. Indeed, these studies find an increase in COT_{min}. It was therefore argued that U_{opt} was traded off by an increase in COT_{min}, allowing preserved migration period in order to reach the spawning area in due time. Therefore, as a strategy, tagged animals may try to swim with the same U_{opt}, but at an increased COT_{min} (Methling et al., 2011).

Equipping New Zealand short-finned eels with a satellite tag would yield a wealth of information about their spawning migration. Additionally, it could potentially be more rewarding than studying species migrating over longer distances, such as the European eel, since the risk of losing the tagged animal is reduced. However, nothing is known about the migration period of this species. Considering the lower U_{crit} and U_{opt} New Zealand short-fin eel as compared to European eel, but similar energetic values (AMR_{crit} and AMR_{opt}, respectively), added drag by a similarly sized tag could increase the associated COT_{min} by factor 3 (Burgerhout et al., 2011). Since the COT_{min} is already higher in New Zealand than in European eels, an increase would come even closer to the measured maximum energetic capacity, expressed as AMR_{crit}. As a result, this increased reduction in scope for activity could lead to a faster depletion of fuel reserves in New Zealand short-finned eel, traveling over the same distance as European eel. However, since anguillid eels are assumed to deplete their energy reserves during migration and subsequent reproduction, without refueling (Tesch, 2003), starvation can be a threat to migrating eels. It therefore was previously suggested that one of the factors determining the onset of the spawning migration are energetic reserves in the form of body fat (van den Thillart et al., 2004).

Additional research is necessary to study the effect of external tracking devises on the swimming capacity of New Zealand short-finned eel.

Methodological Implications

U_{crit} is often defined as the maximum prolonged swimming speed using aerobic and anaerobic metabolism (Plaut, 2001; Blake, 2004), i.e., the swimming speed at which both aerobic and anaerobic exhaustion occurs (Lurman et al., 2008). It is therefore an ecologically significant indicator for the migration capacity of a species. However, recent studies have shown that the results of the U_{crit}-test depends on a variety of experimental factors unrelated to aerobic or anaerobic swimming capacity, including flume length (Kieffer, 2000; Tudorache et al., 2007; Deslauriers and Kieffer, 2012) or post exercise impingement against the rear grid of the swimming tunnel (Tudorache et al., 2010). Additionally, time interval and velocity increment used during the test have been shown to affect U_{crit} (Farlinger and Beamish, 1977; Farrell, 2007). Farlinger and Beamish (1977) showed that with an increase in velocity increments at a fixed time interval, U_{crit} of largemouth bass reached higher values, and with an increase in time intervals at fixed velocity increments, U_{crit} decreased curvilinearly. Farrell (2007) suggests that the duration of the speed increment is important because of a minimum time-interval needed for cardiorespiratory activity to reach a steady state. Even though heart rate can change quickly, cardiac output, blood pressure, and blood gas tensions can take several minutes to reach a steady state at a new speed increment. This allows the following conclusions: firstly, U_{crit}-values are not to be extrapolated to natural conditions, i.e., the U_{crit} only rarely represents the maximum prolonged swimming speed of a freely swimming fish in nature; secondly, in order to apply U_{crit} as an indicator for swimming fitness to compare species or conditions, the U_{crit}-test must be performed using the same experimental parameters. In the present study, oxygen consumption rate ($\dot{M}O_2$) was measured during 60 min time intervals. These long time intervals were necessary for a reliable measurement of oxygen consumption rate with the set up used (van den Thillart et al., 2004). The U_{crit}-test on the other hand, was performed using a time interval of 20 min, in order to compare the results to previous work on swimming eels (Methling et al., 2011; Tudorache et al., 2014), using time intervals of 20 min. Therefore, we chose to measure $\dot{M}O_2$ and U_{crit} in two separate tests with different time intervals.

Similar studies on other species suggested a fitting accuracy (r^2) of more than 0.9 for oxygen measurements over time (Schurmann and Steffensen, 1997; Behrens and Steffensen, 2007), while our results are based on an r^2 of 8.9 ± 2.2. This reduced accuracy in data distribution prevented the measurement of Excess Post-Exercise Oxygen consumption (EPOC), an indicator for the anaerobic capacity of swimming fish (Lee et al., 2003; Svendsen et al., 2010). The Blazka-type set up used in this study is unique with an elongated swimming chamber especially designed for anguilliform swimmers (van den Thillart et al., 2004). The disadvantage is that a relatively large water volume produces more background noise in the measurements and the r^2 therefore is reduced. However, since the results in our study compare with those from previous studies obtained in the same (e.g., Palstra et al., 2008; Burgerhout et al., 2011) or other set ups (e.g., Methling et al., 2011), they can be considered valid. Ideally, respirometry studies on anguilliform swimmers should be conducted using a flume combining an elongated swimming chamber with a low water volume to fish body mass ratio. Similarly, background oxygen consumption by aerobic bacteria can be accountable for a large noise signal in the oxygen measurement. Additional oxygen measurement before and after swimming trials can help to eliminate this potential noise source.

Previous studies suggest correcting for the solid blocking effect (Schurmann and Steffensen, 1997; Methling et al., 2011), while other studies (Jones et al., 1974) claim that a correction is not necessary if cross sectional area of the fish is below 10% of that of the swimming tunnel. Correction for solid blocking effect in the present study resulted in increased water velocities of 5.2 ± 1.6%, which was statistically negligible. However, we advise to perform this correction when data are compared to other laboratory or field studies, because the actual swimming speed could be significantly higher.

The exponential equation used in the present study is based on work by Brett (1964) and Webb (1975) and has been used thereafter on a large variety of fish species. Other studies (e.g., Methling et al., 2011) use a power equation, based on hydrodynamic models. However, there are only two constants to derive in an exponential equation, the SMR and the constant c, which is an inversion of U_{opt}. Since a power function has three constants to derive, the exponential function is more robust. Also, it is more reliable for making predictions beyond the range of measured values (Korsmeyer et al., 2002). This is particularly important for estimating SMR and U_{opt}, crucial values for the interpretation of this study. A power-based equation would tend to overestimate the SMR, because it weighs $\dot{M}O_2$-values at higher swimming speeds more heavily than at lower swimming speeds (Roche et al., 2013). Additionally, a power-based model assumes maintenance costs to remain similar over a range of different swimming speeds, which may not be the case (Farrell and Steffensen, 1987). Considering the high variability of $\dot{M}O_2$ data at high swimming speeds, and the subsequent unreliability of derived and extrapolated data such as SMR and U_{opt}, we chose for the exponential approach. However, whether a model is chosen first and the goodness of fit is calculated subsequently, or the type of model is selected based on goodness of fit, depends on the choice of approach. For a comparative study, the model should be chosen first, in order to calculate how well the data fit the model. For an explorative approach, the model can be chosen based on fit. The present study aimed to compare the swimming physiology of two species; therefore we chose the model first. However, regardless the model chosen for plotting $\dot{M}O_2$, our extrapolated values for SMR are similar to those reported by Methling et al. (2011), who used a power-based model, and our U_{opt}-values represent those by Methling et al. (2011) and Burgerhout et al. (2011) who used even a polynomial model. These similarities in data suggest validity for a larger array of models.

Conclusion

This is the first direct experimental comparison of the swimming capacity of two anguillid eel species, the European eel (*A. anguilla*) and the short-finned eel (*A. australis*). As hypothesized, European eels have a higher U_{crit} and U_{opt}, and a lower COT_{min} than New Zealand short-finned eels, suggesting higher overall swimming capacity, possibly as an adaptation to a longer migration distance. Swimming and energetic parameters obtained in this study can be used for the design and the evaluation of telemetry studies on New Zealand short-finned eels, from a direct comparison with European eel swimming capacity and energetics.

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Swimbladder function and the spawning migration of the European eel *Anguilla anguilla*

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The spawning migration of the European eel is an extensive journey over 5000 to 7000 km from the European coast to the Sargasso Sea. Eels do not feed during this journey and on-board fuels must be sufficient to support the journey of 3.5 to 6 month, as well as sexual maturation and the spawning activity. Swimming of eels appears to be quite energy efficient compared to other fish species, and elevated hydrostatic pressure has been shown to even reduce the costs of transport. Recent studies revealed, however, that during traveling eels perform extensive diurnal migrations and swim at a depth of about 100–300 m at night time, but go down to 600–1000 m at day time. At a depth of 200 m eels are exposed to a hydrostatic pressure of 21 atmospheres (2.13 MPa), while at 800 m hydrostatic pressure increases to 81 atmospheres (8.21 MPa). Accordingly, without any compensation at a depth of 800 m swimbladder volume will be reduced to about 25% of the volume established with neutral buoyancy at 200 m. Consequently, these diurnal changes in depth must be taken into consideration for a calculation of the energy requirements of the spawning migration. Without compensation a compression of the swimbladder will result in a status of negative buoyancy, which makes swimming more costly. Trying to keep the status of neutral buoyancy during descent by gas secretion into the swimbladder in turn requires metabolic activity to enhance swimbladder perfusion and for acid production of the gas gland cells to stimulate gas secretion. During ascent gas is passively removed from the swimbladder in the resorbing section and in the blood transported to the gills, where it is lost into the water. Accordingly, the swimbladder appears to be a crucial organ for the spawning migration. It can be assumed that an impairment of swimbladder function for example due to an infection with the nematode *Anguillicola crassus* significantly threatens the success of the spawning migration.

Keywords: swimbladder function, *rete mirabile*, gas gland cells, European eel, buoyancy, spawning migration

INTRODUCTION

For centuries, the European eel (*Anguilla anguilla*, L) has been an important target species for fishers all over Europe (Tesch, 1999). However, since the 1980s, the stock has been in a steep decline and alarmingly low recruitment numbers are documented in virtually every time series available as well as reflected in landing numbers all over Europe (Dekker, 2003; ICES Advisory Committee, 2013). Nowadays, the European eel stock is considered to be out of safe biological limits and the species is listed in Appendices I–III of the Convention on International Trade in Endangered Species (CITES, 2013).

Reasons currently discussed for this decline are diverse and include exploitation, the loss of habitats, increased mortality due to river obstacles (ICES, 2006) and possible climatic and oceanic changes such as increasing water temperatures in the spawning area, unfavorable wind-driven currents or a shifting of isotherms (Knights, 2003; Friedland et al., 2007; Bonhommeau et al., 2008; Durif et al., 2011; Kettle et al., 2011; Baltazar-Soares et al., 2014). Beside these, habitat and spawner quality are considered major factors influencing recruitment success (Belpaire et al., 2009; Geeraerts and Belpaire, 2010; Clevestam et al., 2011). Due to its complex life cycle *A. anguilla* is specifically vulnerable to environmental changes that potentially impair its ability for long-distance migration, a prerequisite for successful reproduction. To reach its spawning area in the Sargasso Sea (Schmidt, 1923), mature *A. anguilla* have to migrate distances between 5000 and 7000 km, known as the longest spawning migration within the genus *Anguilla* (Aoyama, 2009) and estimated to last between 3.5 and 6 months of continuous swimming (Palstra and van den Thillart, 2010). Animal condition and swimming performance can be severely impaired by a variety of environmental factors like contaminant loads (van Ginneken et al., 2009; Geeraerts and Belpaire, 2010), infection with the introduced swimbladder nematode *Anguillicola crassus* (Kirk, 2003; Palstra et al., 2007; Clevestam et al., 2011) and a lack of energy resources (Svedäng and Wickström, 1997).

Recent attempts to track the spawning migration of the European eel using pop-up satellite archival transmitter tags suggested that the swimbladder as a buoyancy organ may be of special importance during the migration. At night time they travel in the

upper water level at a depth of about 100 to 300 m, while at daytime they prefer deeper water layers between 500 and 700 m, and may even go down to 1000 m (Aarestrup et al., 2009). Although these diurnal migrations typically are not performed in neutral buoyancy at all water levels (Pelster, 1997, 2009, 2013; Sebert et al., 2009b), this observation clearly stresses that a functioning swimbladder is essential and probably indispensable for a successful completion of the spawning migration. During this time eels do not feed and the alimentary canal atrophies (Tesch, 1999). Accordingly, the whole migration culminating in sexual maturity and reproduction must be fueled by on board stores. Several studies tried to obtain an estimate of the energetics of this journey and to relate it to the onboard stores at the onset of the journey (Van den Thillart et al., 2009). Eels appear to have developed a very efficient way of swimming; the cost of transport has been shown to be much lower than in trout, for example. Nevertheless, remaining at a certain water depth is costly for a fish with an overall body density much higher than sea water density, and daily migrations over a depth range of several hundred meters certainly require appropriate adjustments. A functioning swimbladder in this situation significantly contributes to energy saving (Alexander, 1972, 1990; Pelster, 2009, 2013). A reduced swimbladder function in turn will cause an increase in negative buoyancy and in the cost of transport. This most likely will reduce the chances to reach the spawning sites in the Sargasso Sea (Van den Thillart et al., 2009) and thus contribute to a decline in the population of the European eel. Starting with a short description of swimbladder structure and function this paper therefore analyzes how the swimbladder can contribute to vertical migrations and to a successful spawning migration of the eel. This includes a consideration of the possible consequences for the energy requirements for the migration and the impact of an infection of the swimbladder with the nematode *Anguillicola crassus*, which within less than a decade was spread all over Europe. Because at depth oxygen is assumed to be the main swimbladder gas swimbladder tissue is exposed to tremendously high oxygen partial pressures. Therefore, the question how the swimbladder tissue is protected against the formation of reactive oxygen species (ROS) will also be discussed.

SWIMBLADDER STRUCTURE AND FUNCTION

The swimbladder of the eel has extensively been used as a model for swimbladder function in fish because blood is supplied to the eel swimbladder via a bipolar countercurrent system, the so-called "Wundernetz" or *rete mirabile*, which allows for a separate analysis of the functioning of the countercurrent system and of the gas gland cells, which in the eel represent the swimbladder epithelium and are responsible for the initiation of gas secretion (Pelster, 1997, 2009).

Gas molecules simply diffuse along partial pressure gradients from the blood into the swimbladder, and gas secretion therefore is a passive phenomenon. Thus, an initial increase in gas partial pressure is required, the so-called single concentrating effect (Kuhn et al., 1963), which is achieved by a reduction in physical solubility of gases or a decrease in gas carrying capacity of the blood (Root effect). This single concentrating effect is achieved by metabolic activity of the gas gland cells, which produce and secrete lactic acid, even though they are exposed to very high oxygen partial pressures. In the eel swimbladder about 80% of the glucose removed from the blood is converted to lactic acid, and the release of lactate and protons by gas gland cells significantly acidifies the blood (Pelster, 1995). They also produce and release CO_2, mainly generated in the pentose phosphate shunt, i.e., without concomitant consumption of oxygen (Walsh and Milligan, 1993; Pelster et al., 1994). Due to the production of CO_2 in the pentose phosphate shunt a high PCO_2 has to be expected in gas gland cells, driving the outward diffusion of CO_2 into the blood, but also into the swimbladder (**Figure 1**). This contributes to the acidification of blood during passage of the gas gland cells. Accordingly, in the European eel *Anguilla anguilla* blood returning to the *rete mirabile* is significantly acidified after passing the metabolically active gas gland cells (Steen, 1963; Kobayashi et al., 1990b), and this acidification reduces the hemoglobin oxygen carrying capacity via the Root effect (Root, 1931; Pelster and Randall, 1998; Pelster, 2001). **Figure 1** summarizes our current knowledge about the metabolism of gas gland cells and the various mechanisms contributing to the release of protons, lactate and CO_2 from the cells into the blood.

These considerations show that during passage of the gas gland cells the metabolic activity of these cells induces an initial increase in gas partial pressure of all gases in the blood. Depending on the rate of acidification and on the hemoglobin concentration this effect may be very large for oxygen (Pelster, 2001), while for inert gases including nitrogen the salting out effect induced increase in gas partial pressure probably is only quite small due to the comparatively small increase in total solute concentration (Pelster et al., 1988). For PCO_2 also a significant increase can be expected, depending on the activity of the pentose phosphate shunt (Steen, 1963; Kobayashi et al., 1990b; Pelster, 2013).

The increase in gas partial pressure in blood and the increase in PCO_2 in gas gland cells due to metabolic production will generate a pressure head for the diffusion of gas molecules into the swimbladder, but also lay ground for the second step in gas deposition, the multiplication of this initial increase in gas partial pressures by back-diffusion of gas molecules from the venous to the arterial side of the countercurrent system of the swimbladder, the *rete mirabile.* This results in the multiplication of the single concentrating effect in a countercurrent system, so that very high gas partial pressures can be achieved (Kuhn et al., 1963; Kobayashi et al., 1990a; Pelster, 2001, 2009, 2013). **Figure 2** illustrates that in addition to the magnitude of the single concentrating effect (the initial increase in gas partial pressure), the rate of back-diffusion in the countercurrent system, which is dependent on the permeability of membranes and perhaps the presence of special transport proteins, and the length of the *rete* capillaries determine the magnitude of the partial pressure that can be achieved by countercurrent concentration. The initial increase in gas partial pressure measured in the swimbladder is largest for oxygen and therefore oxygen makes up the largest fraction in newly secreted gas, followed by CO_2.

The histological changes of swimbladder tissue of different eel species observed during the preparation of the spawning migration support the conclusion that the swimbladder is of major importance for the journey of the European eel to the Sargasso Sea. Silvering includes a significant enlargement of the

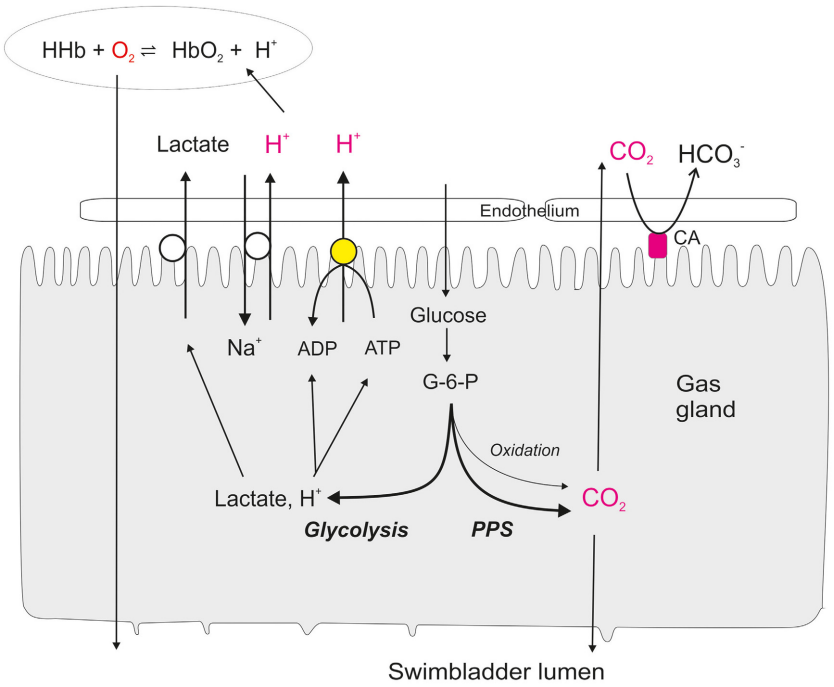

FIGURE 1 | Present concept of glucose metabolism and the secretory activity of swimbladder gas gland tissue. Glucose is taken up from the blood and mainly converted into lactate, in spite of the fact that gas gland tissue typically is exposed to high oxygen partial pressures. A fraction of the glucose is converted to CO_2 in the pentose phosphate shunt (*PPS*). Only a very small fraction of the glucose is oxidized by aerobic metabolism. The CO_2 produced in the cell diffuses down the partial pressure gradient into the swimbladder lumen as well as into the blood. A membrane bound carbonic anhydrase (CA) rapidly establishes the equilibrium between CO_2 and HCO_3^- in the extracellular space and in the blood. Protons are secreted into the blood via a proton ATPase and sodium-proton exchange (NHE). The acidification of the erythrocytes switches on the Root effect and thus reduces the oxygen carrying capacity of the hemoglobin. Oxygen is released from the hemoglobin and diffuses down the partial pressure gradient through the gas gland cells into the swimbladder lumen. Lactate is released into the blood and contributes to the salting out effect.

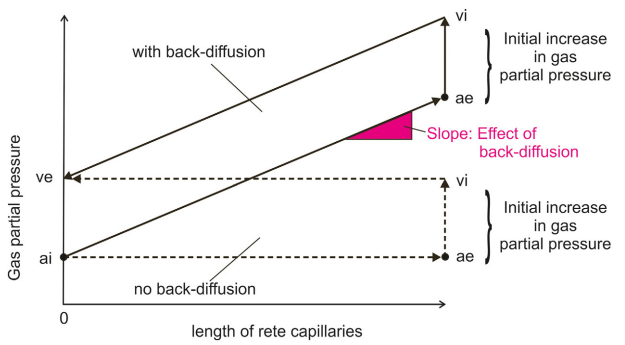

FIGURE 2 | Schema of inert gas partial pressure changes in the *rete mirabile* with and without back-diffusion from the venous to the arterial side of the *rete*. The situation is more complex for oxygen and CO_2, because pH dependent changes in chemical binding will affect partial pressures, and the *rete* is not only permeable to gases, but also to small metabolites. The initial increase in gas partial pressure is brought about by the secretory activity (acid, lactate) of gas gland cells. ai, arterial influx of the *rete mirabile*; ae, arterial efflux; vi, venous influx; ve, venous efflux.

retia mirabilia, indicating an improvement of the countercurrent concentrating ability. In addition, vascularization and swimbladder wall thickness increase as well as guanine deposition in the eel swimbladder wall, which decreases its gas permeability and thus reduces diffusional loss of gas through the swimbladder wall (Kleckner, 1980a,b; Yamada et al., 2001). In the American eel *Anguilla rostrata* a 5-fold increase in the rate of gas deposition has been recorded in silver eels (Kleckner, 1980a). It therefore is assumed that this maturation is connected to a significant improvement in swimbladder function (Sebert et al., 2009b; Righton et al., 2012).

THE SWIMBLADDER DURING VERTICAL MIGRATIONS

Vertical migrations are observed for several fish species with a swimbladder (Marshall, 1972; Vent and Pickwell, 1977; Gee, 1983; Kalish et al., 1986; Neilson and Perry, 1990). Myctophids, for example, are well-known for their daily migrations between the epipelagic zone at night and a depth of 300–700 m during daytime (Watanabe et al., 2001), and also cod has been shown to travel frequently between 50 and 200 m, although the movements do not appear as regular as in Myctophids (Strand et al., 2005). The possible importance of the swimbladder for vertical migrations therefore has been questioned repeatedly and model calculations have been used to predict its possible function. It must be pointed out, however, that experimental data on swimbladder function during vertical migrations are scarce and existing models are based on a number of assumptions, which have not yet been verified. Gas secretion has been measured under atmospheric pressure and silvering has been shown to improve secretion (Kleckner, 1980a),

but how much gas can effectively be secreted at a depth of several hundred meters is unclear. Gas pressure in the swimbladder is higher than the pressure in the surrounding water, so that gas must be lost from the swimbladder simply by diffusion along the partial pressure gradient. Because the partial pressure difference between the swimbladder and the surrounding water increases with depth, this diffusional loss increases with depth. It is known that guanine incrustation for example significantly reduces the gas permeability of the swimbladder wall as compared to other tissues, but it remains unclear how permeable the swimbladder wall is when fish dwell at a depth of several hundred meters. Similarly, gas in contact with an oval or in the resorbing part of the swimbladder will have a much higher partial pressure than the gas in the blood or the surrounding water. Accordingly, gas will be absorbed by the blood along the partial pressure gradient, transported to the gills in the venous circulation and lost into the water. But gas absorption has not been measured at depth and it is not known how much gas can effectively be resorbed. Keeping these uncertainties in mind we still can draw a reasonable picture about the possible role of the swimbladder during vertical migrations, and thus during the spawning migration of the eel.

The increase in hydrostatic pressure with increasing depth compresses the swimbladder, and in a typical teleost the swimbladder wall is not restrained by surrounding tissue. Therefore, swimbladder volume changes with changing hydrostatic pressure according to Boyle's law, except for Cyprinidae, which have rather inextensible walls, probably connected to the role of the swimbladder as an auditory organ (Alexander, 1966, 1972). It is generally assumed that fish are near neutrally buoyant at the upper level of their migration, and negatively buoyant at the lower level (Kanwisher and Ebeling, 1957; Alexander, 1972; Pelster, 2009; Sebert et al., 2009b). There are several good reasons for this. Gas deposition rates recorded so far reveal that gas deposition is a slow process. Bluefish (*Pomatomus saltatrix)* appears to be the fish that deposits gas fastest with about 4 h for filling the swimbladder, but usually it takes about 1 or 2 days for a complete swimbladder filling (Alexander, 1966). Furthermore, if fish with a gas-filled swimbladder were neutrally buoyant at the lower level of their migration the swimbladder would expand during ascent and the fish would rapidly become positively buoyant. Although gas reabsorption is faster than gas deposition, it is limited by blood flow to the resorbing section of the swimbladder and gas transport capacity of the blood and therefore too slow to compensate for the increase in swimbladder volume occurring during a rapid raise over a few hundred meters depth, which often is completed within 1 or 2 h (Strand et al., 2005). American yellow perch (*Perca flavescens)* is able to comfortably compensate a reduction in pressure of 16%, but loses control at a pressure reduction of 32% (Jones, 1952). Accordingly, if fish were neutrally buoyant at the lower level of their distribution range they would be endangered to lose control during a rapid ascent due to the expansion of the swimbladder and the concomitant decrease in overall density.

Considering the diurnal migration of the European eel we can assume an average change in depth between 300 m at nighttime and 800 m during the day (Aarestrup et al., 2009). If we take a 1 kg fish a swimbladder volume of about 50 ml is required to achieve neutral buoyancy (Alexander, 1966, 1971). Hydrostatic pressure at 300 m is 31 atm, therefore a swimbladder volume of 50 ml at this pressure would be equivalent to a volume of 1550 ml at the pressure of 1 atm at the water surface. If the eel then descends to 800 m hydrostatic pressure increases to 81 atm. To remain neutrally buoyant the fish must retain the volume of 50 ml, and according to Boyle's law 50 ml under a pressure of 81 atm would be equivalent to a volume of 4.050 ml at the water surface. Accordingly, the eel would have to secrete 2.500 ml of gas within the 1 or 2 h descent from 300 to 800 m, assuming constant temperature. **Figure 3** shows the amount of oxygen that would be required to keep the volume of the swimbladder constant during the descent in comparison to the oxygen consumption measured in resting and swimming eel. For these calculations it is assumed that at depth the newly secreted gas is almost exclusively oxygen (Alexander, 1966, 1972). Alternatively, a more conservative value of about 60% oxygen in newly secreted gas has been used, which was measured under lab conditions in the European eel (Pelster and Scheid, 1992). Taking a swimbladder perfusion of about 1 $ml*min^{-1}$ and the oxygen carrying capacity of the blood with a hemoglobin concentration of about 5–6 $mmol*L^{-1}$ (Kobayashi et al., 1990b; Pelster and Scheid, 1992) it is obvious that the amount of oxygen required to keep the volume of the swimbladder constant during the descent is orders of magnitude greater than the normal oxygen consumption and orders of magnitude greater than could be supplied by the circulatory system.

Newly secreted gas often contains considerable amounts of CO_2 (Meesters and Nagel, 1935; Copeland, 1952; Wittenberg et al., 1964), and in the European eel CO_2 may make up to 25% of the newly secreted gas (Kobayashi et al., 1990b; Pelster and Scheid, 1992). It is expected that the contribution of CO_2 is reduced at depth, but if the swimbladder volume is to remain constant during vertical migrations gas deposition must be reduced for a couple of hours when swimming constantly at the upper level in order to avoid floating, and then switched on again during the next descent. Gas secretion is initiated by an acidification of the blood during passage of the gas gland cells, and this acidification is achieved by production of lactic acid from glycolysis, and of

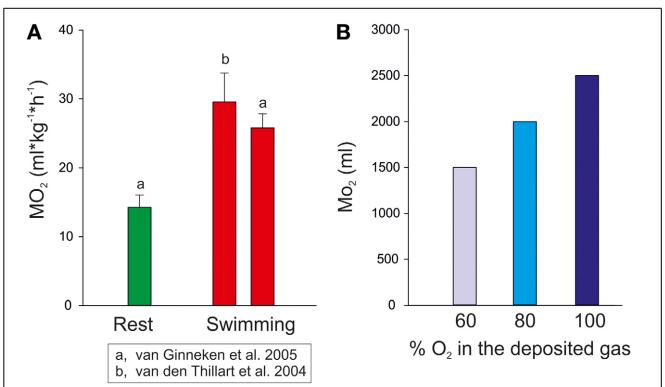

FIGURE 3 | (A) Oxygen consumption of European eels measured at rest and during aerobic exercise (mean ± S.D.) in a swim tunnel (data taken from Van den Thillart et al., 2004 and from van Ginneken et al., 2005). **(B)** The amount of oxygen required for 1 kg eel to keep the swimbladder volume constant at 50 ml when descending from 300 to 800 m.

CO_2, mainly generated in the pentose phosphate shunt (Steen, 1963; Walsh and Milligan, 1993; Pelster et al., 1994; Pelster, 2001). Thus, the initiation of gas secretion does consume glucose for the production of lactic acid and of CO_2, and the rate of gas secretion is related to the glucose consumption by the swimbladder tissue. Under atmospheric pressure about 0.118 mmol*h^{-1} (21.2 mg*h^{-1}) glucose are required for the secretion of 1 ml*h^{-1} of gas in the European eel (Pelster and Scheid, 1993). In a long-term experiment van Ginneken et al. (2005) simulated the spawning migration of the European eel to the Sargasso Sea and observed a decrease in dry matter of 84.3 g*kg^{-1} after 6 month of swimming, compared to 42.7 g*kg^{-1} after 6 month of resting and fasting. Eels used mostly fat for swimming and dry mass carbohydrate content was below 1% at the start and at the end of the 6 month experiment (van Ginneken et al., 2005). From these data we can calculate that a 1 kg eel would not consume more than about 1 to 2 g of carbohydrate for the journey. Accordingly, the amount of carbohydrate consumed in this 6 month swimming experiment but also the onboard carbohydrate stores would not be sufficient to supply the swimbladder with enough glucose to support the required gas deposition. Based on these considerations it appears impossible that the eel will be able to use the swimbladder to retain neutral buoyancy during the observed vertical migrations, and we have to expect that it is near neutrally buoyant only during night time, when it swims in the upper water layers. When descending into deeper layers the eel will become negatively buoyant, and this deficit in buoyancy must be compensated by hydrodynamic lift, i.e., by swimming activity, which in turn requires energy.

ENERGETICS

The energetics of buoyancy and of swimming activity has been extensively studied by Alexander (1966, 1971, 1990). If whole body density of a fish is equal to the density of water the fish has no weight in water, it is neutrally buoyant. If it is denser than water, it is negatively buoyant and will tend to sink. Sea-water density typically is given as 1.026–1.030 kg*L^{-1}, and the density of fish usually is ~1.08–1.10 kg*L^{-1}. To achieve neutral buoyancy, this weight must be balanced by lift. Thus, the lift (L) required is

$$L = V^{*}g^{*}(\rho_b - \rho_w), \quad (1)$$

where V is the volume, g is the gravity force, ρ_b is the density of the fish, and ρ_w is the density of sea-water. From these considerations it can be calculated that in sea-water a 1 kg fish will achieve neutral buoyancy with a swimbladder volume of ~50 ml.

Swimming will generate hydrodynamic lift, mainly at the pectoral fins, which are used as hydrofoils, but depending on the structure the peduncular keel may also contribute to the generation of lift (Alexander, 1972; Magnuson, 1978; Gee, 1983). The generation of hydrodynamic lift by swimming, however, induces drag, and work must be done against the drag. If we assume that during a vertical migration at the deeper level the swimbladder volume will be too small to provide neutral buoyancy, the fish needs hydrodynamic lift to compensate for this deficit in order to keep his position in the water column. **Figure 4** shows the additional lift required by the eel assuming that it descends from the upper level of 200 or 300 m without keeping the swimbladder volume constant, i.e., with decreasing volume of the swimbladder proportional to the increase in hydrostatic pressure at constant temperature. About 6 Newton will be required to compensate for the negative buoyancy a 1 kg fish will encounter at a depth of 800 m, if it moved down from a depth of 300 m, starting with neutral buoyancy.

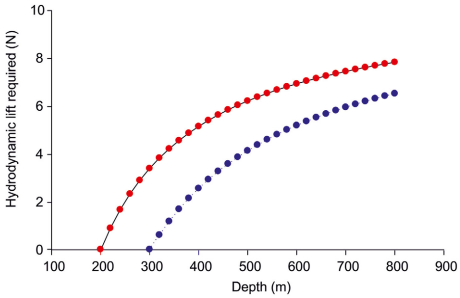

FIGURE 4 | The lift (in N) required for a 1 kg eel when descending from 200 (red) or 300 m (blue) down to 800 m without compensatory gas secretion, i.e., the swimbladder volume decreases according to Boyle's law.

Assuming hydrodynamic lift is mainly produced at the fins, the work required for this swimming activity can be estimated as:

$$W = T * (P_2 - P_1) * V * \rho * g * U * D * P_2^{-1}, \quad (2)$$

where W is the work required if the fish encounters this drag for a certain time (T), P_1, and P_2 are the hydrostatic pressures at the two different depth levels, V is volume, ρ is the density, g is gravity force, U is swimming speed, and D is an estimate of the extra drag the eel must suffer to obtain the extra lift (Alexander, 1971).

For the extra drag a fish must suffer to obtain the extra lift Alexander (1971) assumed a value of 0.2. If we take this value, which is dependent on the Reynolds numbers of the fins (Alexander, 1972), a swimming speed of 0.35–0.45 m*s^{-1}, which is required to reach the Sargasso Sea in time (see below) we can calculate the work required during the time period of 12 h for the descent and the stay at the lower depth. The results of this calculation are shown in **Figure 5**, assuming that a 1 kg eel starts to move down from a depth of either 200 or 300 m with a swimbladder volume of 50 ml and neutral buoyancy, and the decreasing swimbladder volume is not compensated by gas secretion. Depending on the swimming speed the descent down to 800 m will require about 1.0–1.4 kJ for a 1 kg fish within 12 h.

This calculation was originally developed for a "typical" fish, and the elongated eel body looks quite different. Eels do not have a peduncular keel, and this structure therefore cannot contribute to the generation of lift, but the body itself and undulatory movements may influence the generation of hydrodynamic lift (Magnuson, 1970, 1978), but also the level of drag encountered during swimming. Accordingly, the actual values for the eel may slightly deviate from those calculated here, but it is obvious that a significant amount of work must be spent for hydrodynamic lift generation, and this work increases with increasing distance between the upper and the lower level of the diurnal migration.

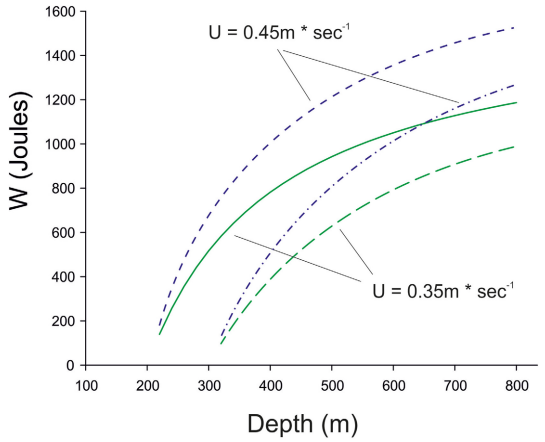

FIGURE 5 | The work required for a 1 kg eel when descending for 12 h from a depth of 200 or 300 m down to 800 m without compensatory gas secretion, i.e., the swimbladder volume decreases according to Boyle's law. Swimming speed was assumed to be either 0.35 $m*s^{-1}$ (green) or 0.45 $m*s^{-1}$, (blue), which is required to reach the Sargasso Sea within a period of 6 month. Long-term swim tunnel experiments have shown that eels with a body length of about 0.8 m can swim for several month at a speed of 0.50 $BL*s^{-1}$, which would be equivalent to 0.40 $m*s^{-1}$ (Van den Thillart et al., 2004; van Ginneken et al., 2005).

While this consideration assumes that the fish swim in order to stay at a certain water depth, eels must swim anyway in order to reach their spawning ground. Tracking experiments following 11 silver eels in the North Sea revealed a swimming speed of 0.69–0.96 $cm*s^{-1}$, equivalent to 0.6–0.9 $Bl*s^{-1}$ (Tesch, 1974; Beamish, 1978). Using long-term swimming experiments the spawning migration was imitated in the lab and silver eels were swum for 3 or for 6 month in a swim tunnel (Van den Thillart et al., 2004; van Ginneken et al., 2005). The swimming speed was set to 0.39 $m*s^{-1}$ and to 0.36 $m*s^{-1}$ (= 0.5 $Bl*s^{-1}$), so that the eels would cover a distance of 5.500 km within 6 month. These experiments demonstrated that eels are able to cover the distance from the European coast to the Sargasso Sea with their on board energy reserves without food intake, and based on the oxygen consumption and on bomb-calorimetry an energy consumption of about 0.42–0.83 $kJ*kg^{-1}*km^{-1}$ was calculated. This swimming speed results in a daily migration of about 30–35 km, accordingly within 12 h the energy consumption amounts to 6.3–14.5 kJ for a 1 kg eel swimming under atmospheric pressure with a status of neutral buoyancy. Accordingly, the energy required to compensate for the decrease in buoyancy during the decent amounts to about 22% of the energy required for swimming with neutral buoyancy in the worst case, and 7% as a minimum estimate. If we assume that in the upper depth range (200–300 m) eels swim more or less in a status of neutral buoyancy the required energy expenditure should be comparable to the values obtained in the swim tunnel experiment.

As already mentioned these calculations are based on a number of assumptions and therefore must be taken as estimates. For the eel in particular it appears possible that the actual energy expenditure required for swimming is lower than calculated, and at depth it may also be lower than measured in the swim tunnel experiments. Swimming performance and slow muscle power output have been shown to be improved in silver eels as compared to yellow eels (Ellerby et al., 2001; Quintella et al., 2010), and it could be that in the *in vivo* situation in migrating eels this improvement is even enhanced. Furthermore, silver eels under pressure swim more efficient than under atmospheric pressure: Oxygen consumption of male silver eels measured at swimming speeds between 0.2 and 1.0 $Bl*s^{-1}$ at a pressure of 1 atm and then at 101 atm was significantly lower at high pressure (Sebert et al., 2009a). If this can be transferred to the *in vivo* situation of migrating eels the actual amount of energy required for swimming would be lower than measured in the swim tunnel experiments (Van den Thillart et al., 2004; van Ginneken et al., 2005). This would increase the relative amount of energy required for buoyancy compensation during vertical migrations, but it is unknown how the repeated changes in hydrostatic pressure affect the energy requirements for swimming activity. It therefore would very interesting and important to get a better insight into the swimming efficiency of eels during their spawning migration and on the effect of the repeatedly changing hydrostatic pressure on swimming performance.

Vertical migrations cause a change in hydrostatic pressure and therefore affect the buoyancy status of fish with a compressible swimbladder, but they also increase the distance covered by the fish. From the traces recorded in migrating eels it can be estimated that the descent from 200 to 300 m down to 700–800 m takes about 1.5 to 2.5 h, and ascending to the higher level during the night takes about the same time (Aarestrup et al., 2009). If the change in depth would be achieved by vertical movements, a change in depth of 500 m would increase the distance by 1 km per day. Based on the distance between the European coast and the Sargasso Sea and the time required to cover this distance it can be calculated that eels must swim about 30–35 km per day. If eels would travel at their optimal swimming speed of 0.61–0.68 $m*s^{-1}$ (= 0.74–1.02 $Bl*s^{-1}$) with a minimum energy expenditure (Palstra et al., 2008) they would even swim more than 40 km per day and reach the Sargasso Sea in less than 5 month. This means, that the vertical migration can at most increase the distance to be covered every day by about 3%, and because eels do not swim vertically it is far less than 3% and therefore probably negligible.

Nevertheless, the vertical migrations increase the energy required for the spawning migration and the question remains, why they are performed. Aarestrup et al. (2009) hypothesized that the vertical migrations are connected to thermoregulatory behavior. The descent to cooler water was supposed to keep average temperature below 11°C, delaying gonadal development, which is only completed towards the end of the spawning migration. Overall metabolic rate also decreases with decreasing temperature, thus the descent may help to reduce energy expenditure. The overall temperature differences between the upper and the lower water level, however, was only slightly above 1°C (Aarestrup et al., 2009), so that other effects may play a role. Most likely predator avoidance may contribute to the daytime descent (Schabetsberger et al., 2013). Studies using popup satellite tags reveal that there is predation on migrating European and American eels, not only

near the coast, but also in the open ocean (Béguer-Pon et al., 2012; Westerberg et al., 2014).

ROS

Because oxygen makes up a large fraction of the swimbladder gas at depth, the high hydrostatic pressures encountered during the vertical migrations must result in very high oxygen partial pressures (several ten or may be hundred atmospheres) in the swimbladder and thus in gas gland cells (Fänge, 1983; Kobayashi et al., 1990b; Pelster, 2009). This leads to another interesting question and important topic: How does the gas gland tissue protect itself from oxygen damage caused by ROS, which are typically generated at high oxygen tensions (Brueckl et al., 2006; Valko et al., 2007; Alfadda and Sallam, 2012)? In the mammalian lung hyperoxic ventilation causes production of O_2^- and H_2O_2, which for example, activate endothelial cells contributing to lung injury, and stimulate inflammatory reactions (Chabot et al., 1998; Brueckl et al., 2006). Mitochondria, the main production site for ROS, are not numerous but present in gas gland cells (Dorn, 1961; Pelster, 1995), and the membrane bound enzyme NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) may also generate superoxide and thus contribute to ROS production. At a PO_2 of many atmospheres the generation of ROS therefore must be expected.

Our preliminary studies revealed the presence of glutathione reductase activity in gas gland cells of the European eel, while it was not detected in other tissues, and also superoxide dismutase and catalase activity were found in homogenates of eel gas gland tissue, and these enzymes are important for the degradation of ROS (Schneebauer and Pelster, unpublished results). In several marine species activities of these enzymes in swimbladder tissue appear to be higher than in other tissues, but the activity was not correlated to an inflation or deflation of the swimbladder (Morris and Albright, 1981, 1984). The activity of the pentose phosphate shunt, which is important for the CO_2 production of gas gland cells (see above), can also be seen in this context. In this shunt $NADPH+H^+$ is generated, which is used by radical oxidizing enzymes like glutathione reductase for the detoxification of ROS. While eels in freshwater with a limited water depth probably hardly experience an oxygen partial pressure of more than a few atmospheres, during the spawning migration the expected oxygen partial pressures are many times higher. Accordingly, it can be expected that the silvering process includes a significant increase in the capacity of the swimbladder tissue to deal with ROS in order to avoid tissue damage. A detailed analysis of the oxygen defense systems in silver eels therefore appears to be quite promising and may provide interesting insights into the mechanisms preventing tissue damage due to ROS production, or, alternatively, elucidate how excessive ROS production can be prevented in the presence of high oxygen partial pressures.

ANGUILLICOLA

The recent decline in the population of the European eel may in part be related to an infection of the swimbladder with the nematode parasite *Anguillicola crassus* (= *Anguillicoloides crassus*). This sanguivorous, histotrophic nematode was brought to Europe in the 1980's and within a decade a large fraction of the European eels was infected (Moravec, 1992; Schabuss et al., 2005). An infection of the swimbladder results in severe alterations of the swimbladder epithelium (Nimeth et al., 2000; Würtz and Taraschewski, 2000), and based on the macroscopical appearance of the swimbladder and the exudates present in the bladder an infection dependent degeneration of the swimbladder was recently confirmed (Lefebvre et al., 2013). Given the importance of gas gland cell metabolism for the initiation of gas secretion these histological alterations of the swimbladder tissue, which include the formation of a multilayered epithelium with an increase in the diffusion distance between the blood and the swimbladder lumen, was expected to cause an impairment of swimbladder function. A detailed analysis of the swimbladder gas composition and of the rate of gas secretion in relation to the level of infection indeed revealed a significant impairment of swimbladder function by this nematode (Würtz et al., 1996). The rate of gas secretion was significantly reduced in infected eels, and the fraction of oxygen within the newly secreted gas was significantly lower than in uninfected eels (**Figure 6**), suggesting that the blood acidification by the gas gland cells and the countercurrent concentrating ability was impaired by the nematode. This is in line with the observation that infected eels have a lower number of circulating erythrocytes and thus a reduced oxygen carrying capacity in their blood (Boon et al., 1990).

In addition, swim tunnel experiments showed that infected silver eels have a lower cruising speed, and the costs of transport were elevated by about 20%. Almost 50% of the eels with a heavily infected swimbladder stopped swimming at comparatively

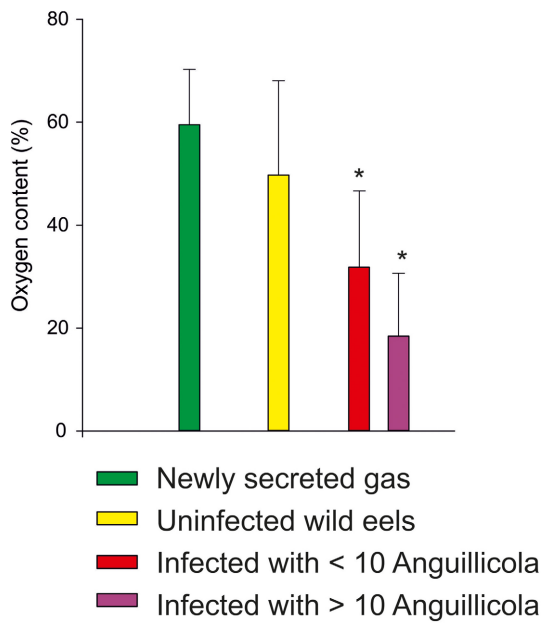

FIGURE 6 | Swimbladder oxygen content measured in lab experiments (green, Kobayashi et al., 1990b) and in swimbladder gas samples taken from wild eels not infected with *Anguillicola crassus*, and from eels infected or heavily infected with the nematode (data from Würtz et al., 1996). Mean ± S.D.; * indicates significant differences between uninfected and infected wild eels, $p < 0.05$, Kruskall–Wallis-test.

low swimming speeds, and in a long term swimming experiment mimicking the spawning migration infected eels showed an early migration failure (Palstra et al., 2007). Taken together these data demonstrate that the reduced swimbladder function due to an infection with *Anguillicola crassus* impairs swimming performance of the eel and thus increases the energy demand for the spawning journey. A recent study suggested that an artificial infection of eels with the nematode may advance the silvering process (Fazio et al., 2012). Given the thickening of the swimbladder epithelium and the reduction in gas deposition in response to an infection, however, it is expected that in infected eels the silvering related adaptations of gas gland cell physiology cannot occur as to be expected for an uninfected swimbladder, and the elasticity of the swimbladder wall of infected eels has been shown to be significantly reduced as compared to uninfected eels (Barry et al., 2014). The results so far clearly suggest that the infection with the nematode will impair the spawning migration to the Sargasso Sea, and as a worst case scenario it might even make a successful spawning migration impossible.

PERSPECTIVES

The fish swimbladder and in particular the eel swimbladder and its role during the spawning migration has fascinated scientist for more than a hundred years, but it still remains a mystery. Recent molecular studies and experiments trying to artificially induce silvering provided significant insight and indicate that silvering is more like the onset of puberty than a genuine metamorphosis (Aroua et al., 2005). The molecular changes in gas gland tissue, however, associated with the process of silvering have not been analyzed so far. Given the importance of the swimbladder during the spawning migration this appears to be a promising area, and first studies are underway. In this context it also would be interesting to see how the tissue is able to avoid damage caused by ROS or alternatively, how the tissue is able to avoid the generation of ROS in the presence of hyperbaric oxygen pressures.

While the first swim tunnel experiments have been performed under constant pressure and therefore in a status of neutral buoyancy, in order to better understand the spawning migration it would be necessary to analyze the swimming performance and energetics under conditions of variable negative buoyance as to be expected in the migrating eel. Although the first swim tunnel experiments suggest that the swimbladder nematode *Anguillicola crassus* impairs swimming performance, it is still unclear whether eels with an infection or eels suffering from a previous infection will be able to successfully migrate to the Sargasso Sea, and how the infection affects the silvering process in the swimbladder. Our knowledge about swimbladder function in the eel and the spawning migration has been significantly advanced over the last hundred years, but many questions remain. Answers to these questions are necessary to better understand population dynamics of the European eel and to be able to find appropriate means to stabilize the currently declining populations.

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