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How the expression of green fluorescent protein and human cardiac actin in the heart influences cardiac function and aerobic performance in zebrafish *Danio rerio*

S. R. Avey*, M. Ojehomon‡, J. F. Dawson‡ and T. E. Gillis*†

*Department of Integrative Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada and ‡Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada

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The present study examined how the expression of enhanced green fluorescent protein (eGFP) and human cardiac actin (ACTC) in zebrafish *Danio rerio* influences embryonic heart rate (R_H) and the swim performance and metabolic rate of adult fish. Experiments with the adults involved determining the critical swimming speed (U_{crit}, the highest speed sustainable and measure of aerobic capacity) while measuring oxygen consumption. Two different transgenic *D. rerio* lines were examined: one expressed eGFP in the heart (*tg(cmlc:egfp)*), while the second expressed ACTC in the heart and eGFP throughout the body (*tg(cmlc:actc,ba:egfp)*). It was found that R_H was significantly lower in the *tg(cmlc:actc,ba:egfp)* embryos 4 days post-fertilization compared to wild-type (WT) and *tg(cmlc:egfp)*. The swim experiments demonstrated that there was no significant difference in U_{crit} between the transgenic lines and the wild-type fish, but metabolic rate and cost of transport (oxygen used to travel a set distance) was nearly two-fold higher in the *tg(cmlc:actc,ba:egfp)* fish compared to WT at their respective U_{crit}. These results suggest that the expression of ACTC in the *D. rerio* heart and the expression of eGFP throughout the animal, alters cardiac function in the embryo and reduces the aerobic efficiency of the animal at high levels of activity.

Key words: cost of transport; critical swimming speed; heart rate; O_2 consumption; transgenic protein expression.

INTRODUCTION

Zebrafish, *Danio rerio,* (Hamilton 1822) have become an increasingly common model for the study of congenital diseases of skeletal muscle and the cardiovascular system. This species is amenable to genetic manipulation and there is significant conservation in gene sequence and protein function between humans and *D. rerio*. As a result, human mutant genes are being incorporated into the genomic DNA of *D. rerio* to examine the molecular basis of disease dysfunction (Ha *et al*., 2013; Buhrdel *et al*., 2015; Bragato *et al*., 2016; Linsley *et al*., 2017). To rapidly screen for the expression

†Author to whom correspondence should be addressed. Tel.: +1 519 824 4120, extn 58786; email: tgillis@uoguelph.ca

of non-native proteins in the transparent *D. rerio* embryo, the complementary (c)DNA for green fluorescent protein (GFP) driven by an expression promoter is co-injected into cells (Long *et al*., 1997; Brion *et al*., 2012). Although the literature suggests that enhanced (e)GFP can be used with minimum perturbation of cellular function (Feng *et al*., 2000), there have been reports where that is not the case. For example, targeted expression of eGFP in the heart can cause fatal cardiomyopathy in common mice *Mus musculus* and can also interfere with excitation-contraction coupling by interacting with myosin (Huang *et al*., 2000; Agbulut *et al*., 2006). This indicates that eGFP expression in the heart affects cardiac function. Therefore, explicit experimental validation is needed to determine if incorporating eGFP into the genomic DNA of an organism, or the expression of the resultant protein, will affect physiological processes, such as oxygen metabolism or muscle contraction.

The purpose of this study was to investigate the potential effects of eGFP transgenes on heart rate in *D. rerio* embryos and swim performance, oxygen consumption and bioenergetics in adult male *D. rerio*. The study examined two transgenic *D. rerio* lines. The first expressed eGFP in the heart (*tg(cmlc:egfp)*) and the second expressed human actin in the *D. rerio* heart and eGFP throughout the body (*tg(cmlc:actc,ba:egfp)*). Human cardiac actin (ACTC) was used as this would be the control for any study examining the consequence of actin mutants on the function of the heart or skeletal muscle. It was predicted that indicators of cardiac function and aerobic performance would be affected in the *D. rerio* expressing eGFP throughout the heart (*tg(cmlc:egfp)*) as well as in the *D. rerio* expressing eGFP throughout the organism and human ACTC in the heart (*tg(cmlc:actc,ba:egfp)*).

MATERIALS AND METHODS

PLASMIDS

The Tol2 transposon vector consisting of the GFP gene expressed by the cardiac myosin light chain 2 (cmlc2*)* promoter (pKTol2cmlc-GFP) and the β-Actin (BA) promoter in a construct with a cmlc promoter ready to insert a gene of interest (pKTol2cmlcBA-GFP) was subcloned into the pKTol2cmlcBA-GFP vector behind the *cmlc2* promoter by a subcloning company, Mutagenex (www.mutagenix.com). This cloned plasmid is called WT pKTol2cmlc-actc BA-GFP.

MESSENGER RNA PRODUCTION

PT3TS-Tol2 plasmid was transformed into NovaBlue competent cells (www.novagen.com) and plasmid preparations were then isolated, quantified, linearized with BamHI (He *et al*., 2009) and purified using a QIA PCR Purification kit (QIAGEN; www.qiagen.com). The digested plasmid was run on a 0·6% agarose gel to verify digestion and purity. After linearization, the DNA was transcribed to messenger (m)RNA using a mMessage T3 kit (ThermoFisher; www .thermofisher.com) (He *et al*., 2009). The RNA was then precipitated and resuspended in 12 μl of milliQ water (Merck Millipore; www.merckmillipore.com) and quantified using a Nanodrop spectrophotometer (www.nanodrop.org). Purity was checked according to Clark *et al*. (2011).

EGG COLLECTION

WT *D. rerio*, purchased from AQUAlity Tropical Fish Wholesale, (Mississauga, ON, Canada) were maintained in the Hagen Aqualab at the University of Guelph, ON, Canada, on a 14 h light schedule at approximately 28° C. Fertilized eggs were obtained using previously described methods (Nelson & Van Der Kraak, 2010). All work completed in this

study, associated with animals, was approved by the University of Guelph's Animal Care Committee under the auspices of the Canadian Council for Animal Care (Animal Use Protocol #3407).

TRANSGENE PREPARATION AND INJECTION

All injections were into the yolk of the egg at the one cell stage. The needles used for the injections were made from 1·0 mm outer diameter glass capillary tubes using a Flaming Brown Micropipette Puller (Sutter Instruments; www.sutter.com). Using an Eppendorf microloader pipette (www.eppendorf.com), the needle was back-loaded with at least 1·5 μl of the injection material. A #5 Dumont Donostar forcep (FST; www.finescience.ca) was used to clip the needle to obtain the right bolus size. To create the *tg(cmlc:egfp)* line, the transposase mRNA (12 ng μl^{-1}) and pKTol2cmlc-GFP (12 ng μl^{-1}) were injected into each *D. rerio* embryo. To create the *tg(cmlc:actc,ba:egfp)* line, transposase mRNA (6 ng μl^{-1}) and WT pKTol2cmlc-ACTC-BA-GFP (6 ng μl^{-1}) were injected. Bolus size was 10 nm. Following injection, the embryos recovered in an incubator (ThermoFisher) set to 28° C for a minimum of 15 min. The eggs were then transferred to a clean Petri dish and kept in the incubator set to 28° C. The developing embryos were checked daily using a dissecting microscope (Carl Zeiss; www.zeiss.com) and any deformed embryos were removed. The expression of eGFP in the embryos was confirmed at 24 h post-fertilization (hpf) for the *tg(cmlc:actc,ba:egfp)* and at 48 hpf for the *tg(cmlc:egfp)* using a dissecting microscope equipped with a GFP filter. *tg(cmlc:actc,ba:egfp)* embryos had fluorescence throughout the body and the *tg(cmlc:egfp)* embryos had fluorescence in the heart [Fig. 1(a), (b)].

GERM-LINE TRANSMISSION

Germ-line transmission was carried out according to the procedure described by Huang *et al*. (2003). Transgenic *D. rerio* embryos displaying either a fluorescent heart or a fluorescent body were separated from WT and raised to adulthood. Pairs of male and female founders were initially mated to screen for transgenic *D. rerio*. If GFP expression was found in some of the resulting embryos, the founders were separated and allowed to mate with WT to identify the germ-line transmitting parent.

EMBRYO HEART RATE MEASUREMENTS

Videos clips of transgenic and WT embryos in separate Petri dishes were taken for 15 s using a dissecting microscope equipped with an eyepiece adaptor (Carson's; www.carson.com) using an iPhone5S camera (Apple Corp.;www.apple.com) recording at 1080 p resolution and 30 frames s^{-1}. Moving pictures experts' group (MPEG) Streamclip software (www.mpeg-streamclip.wondershare.net) was used to convert the video clips from QuickTime (Apple Corp.) movie (MOV) files to audio video interleave (AVI) files. Using Fiji software (Schindelin *et al*., 2012) the heart wall movement was tracked within a drawn window and a periodogram from the z-stack profile produced. Spectral analysis of the periodogram using online resourses (www.wessa.net/rwasp_spectrum.wasp) resulted in the best fit harmonic (H), which is the number of heart beats in the length of video clip (L_V, s). The heart rate (R_H) in beats min^{-1} (bpm) was calculated using the equation: $R_H = (HL_V^{-1})60$ s.

FISH MATURATION AND MAINTENANCE

At 24 hpf embryos were sterilized using 0·07% bleach and dechrionated manually. Non-deformed embryos that survived at 5 dpf were transferred to bigger aquaria. Adult male WT, *tg(cmlc:egfp)* and *tg(cmlc:actc,ba:egfp)* lines were kept in separate 2 l recirculating tanks in the Hagen Aqualab. Fish were held at a maximum density of 10 fish per tank with water held at 28° C and the room was kept on a 14 h light schedule. Fish were fed *ad libitum* with brine shrimp and TetraFin goldfish flakes (www.tetra-fish.com).

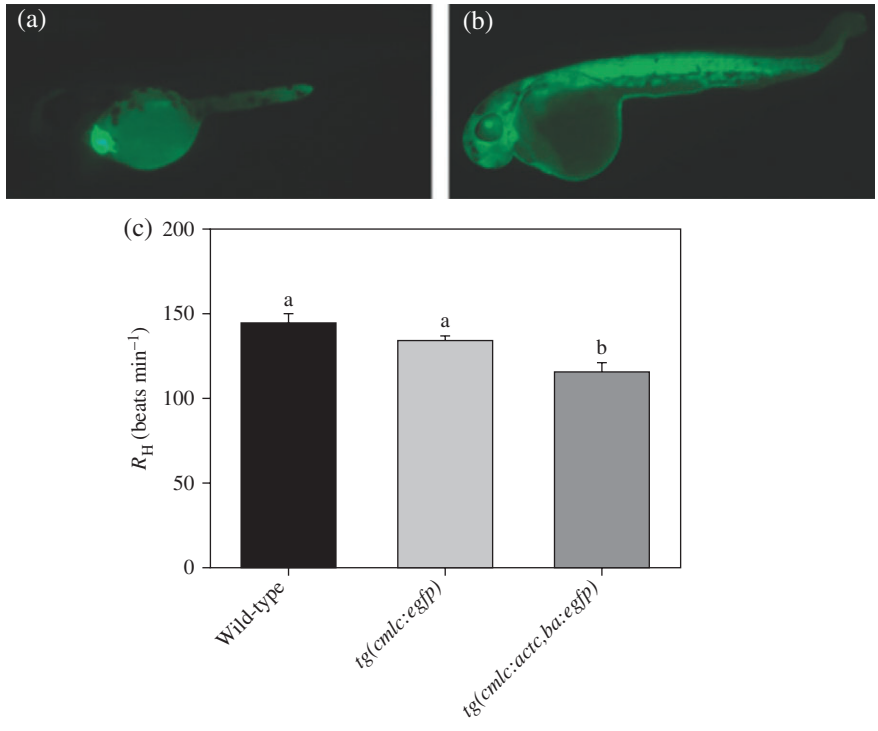

FIG. 1. (a) *Danio rerio* 2 days post fertilization (dpf) *tg(cmlc:egfp)* embryos expressing eGFP in the heart; (b) *tg(cmlc:actc,ba:egfp)* embryo 2 dpf expressing human ACTC protein in the heart and eGFP throughout the body; (c) mean ± S.E. heart rate (R_H) measurements of wild-type ($n = 24$), *tg(cmlc:egfp)* ($n = 22$), and *tg(cmlc:actc,ba:egfp)* ($n = 30$) *D. rerio* embryos 4 dpf at 28° C. Bars that do not share a common letter are significantly different from each other ($P < 0{\cdot}05$).

METABOLIC RATE AND CRITICAL SWIMMING SPEED

Swim tests and respirometry measurements of all *D. rerio* lines were completed using a 170 ml swim tunnel system, equipped with Witrox, AutoResp software (Loligo Systems; www.loligosystems.com), fibre optic oxygen probe (PreSens Precision Sensing GmbH; www.preSens.de) and DAQ-M automated oxygen measurement system (NationalInstruments Co.; www.ni.com). The oxygen probe was routinely calibrated throughout the study at 0 and 100% air saturation at 28° C using Witrox. The temperature within the swim tunnel was kept constant at 28 ± 0·3° C and the level of oxygen saturation was maintained using an airstone. Before each experiment a single fish was transferred to the swim tunnel and allowed to acclimate for 3 h to a speed of 1·0 body lengths (L_B) s^{-1}. Oxygen consumption measurements made during this acclimation period demonstrated that metabolic rate had stabilized before the 3 h mark (Fig. S1, Supporting information). After the acclimation period, water velocity was manually increased by 1·0 L_B s^{-1} every 5 min. Experiments were completed when individuals could no longer hold their position in the current and rested on the mesh at the back of the swim tunnel for 5 s. The final swimming speed achieved prior to fatigue is defined as the maximal sustainable swimming speed (U_{crit}), defined by the equation: $U_{crit} = V_{ls} + (t_s t_i^{-1} V_i)$, where V_{ls} is the final speed (cm s^{-1}) completed, t_s is the time spent at the final speed, t_i is the time increment (min) and V_i is the speed increment (Plaut, 2001).

Oxygen consumption measurements during the swim tests were made using a standard stop flow method (Masse *et al*., 2013). The amount of dissolved oxygen in the swim tunnel was measured and used to calculate the mass-specific metabolic rate (μmol g^{-1} h^{-1}) of each fish using the equation: $M_{O_2} = (\Delta O_2 \Delta t^{-1})[60\,\text{min(h)}^{-1}][(v - m_w)m^{-1}][(1\,\text{mmol}\ O_2)(32\,\text{mg}\ O_2)]1000\,\mu\text{mol mmol}^{-1}$, where ΔO_2 is the change in oxygen content of the water (mg l^{-1}) over Δt (3 min), v is the volume of the swim tunnel (0·17 l), m is the wet mass of the fish (g) and m_w is the amount of water displaced by the fish (1 g = 0·001 l). During the stop–flow test, dissolved oxygen was never allowed to decrease below 100%. Wet mass was used in these calculations so that the fish did not have to be sacrificed (Vergauwen *et al*., 2013; Gerger *et al*., 2015). This was necessary as these fish were being used to maintain the transgenic lines. All fish were blotted dry before weighing.

COST OF TRANSPORT

The cost of transport (C_T) determines the energy required for a fish to move a unit of distance (Masse *et al*., 2013), representing the energetic cost to maintain the standard metabolic rate and swim at a particular water velocity (Videler, 1993). This value therefore provides an index of swimming efficiency, with a lower C_T value representing more efficient locomotion (Claireaux *et al*., 2006). The following equation was used to calculate the C_T (J kg^{-1} m^{-1}) for individual fish at each velocity: $C_T = \{(\Delta O_2 \Delta t^{-1})[60\,\text{min(h)}^{-1}][(v - m_w)m^{-1}][1000\,\text{g(kg)}^{-1}][\text{h}(3600\,\text{s})^{-1}]\ 14{\cdot}1\,\text{J mgO}_2{}^{-1}\}$.

STATISTICAL ANALYSIS

Shapiro–Wilk and *F*-tests were performed using Microsoft Office Excel 2007 on the U_{crit} values and all metabolic rate measurements, to examine normality and homogeneity of variance, respectively. Log_{10} transformations were carried out on data that did not meet the assumptions. One-way ANOVAs with post-hoc Tukey honest significant difference were performed using Online Web Statistical Calculators (www.astatsa.com) on the morphological data, heart rate measurements and the U_{crit} values. A one-way ANCOVA with post-hoc Bonferroni correction was performed using MedCalc 17·6 (MedCalc; www.medcalc.org), on the metabolic rate and cost of transport data at U_{crit} with body condition as the covariate. Body condition was used as the covariate as it takes both body length (L_B) and body mass (m) into account. All assumptions of the test were met. All statistical tests used a significance level of $P = 0{\cdot}05$. Data are shown as mean ± S.E.

RESULTS

HEART RATE

At 4 dpf the heart rates of *tg(cmlc:actc,ba:egfp)* fish were 20% lower than the WT fish and 14% lower than the *tg(cmlc:egfp)* fish ($P < 0{\cdot}01$ and $P < 0{\cdot}05$, respectively). There was no significant difference in the heart rates of the embryos from the WT and *tg(cmlc:egfp)* fish [Fig. 1(c)]. Heart rate was not measured on any subsequent days as this would have required that the fish be restrained, with possible consequences from handling stressors.

U_{CRIT} AND METABOLIC RATE

There was no significant difference in the U_{crit} of any of the three experimental groups (WT, *tg(cmlc:egfp)*, *tg(cmlc:actc,ba:egfp)*) [$P > 0{\cdot}05$; Fig. 2(a)]. These values were 7·45 L_B s^{-1}, 8·54 L_B s^{-1} and 7·74 L_B s^{-1} for the WT fish, *tg(cmlc:egfp)*

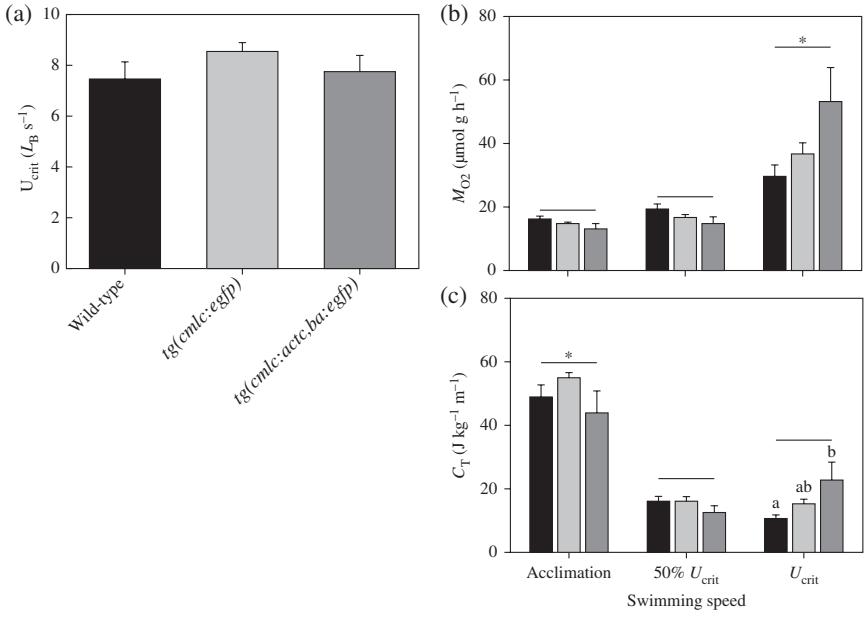

FIG. 2. The effect of transgene expression [■, wild type, $n = 10$; □, *tg(cmlc:egfp)*, $n = 9$; ■, *tg(cmlc:actc,ba:egfp)*, $n = 5$] on: (a) mean ± S.E. the critical swimming speed (U_{crit}) at 28° C in adult male *Danio rerio*; (b) mean ± S.E. *w*et mass-specific oxygen consumption rates (M_{O_2}) of adult male *D. rerio* swimming at the acclimation speed [1 body length (L_B) s^{-1}], at the speeds required to achieve 50% U_{crit}, and U_{crit} at 28° C; (c) mean ± S.E. the cost of transport (C_T) for adult male *D. rerio*, swimming at the acclimation speed (1 L_B s^{-1}), at the speeds required to achieve 50% U_{crit}, and U_{crit} at 28° C. For all characteristics, N (number of fish) = 10 for WT; 9 for *Tg(cmlc:eGFP)*; and 5 for *Tg(cmlc:ACTC,BA:eGFP)*. * indicates a significant effect of swimming speed, while bars that do not share a common letter are significantly different from each other ($P < 0{\cdot}05$).

fish and *tg(cmlc:actc,ba:egfp)* fish, respectively. At U_{crit}, the metabolic rate of the *tg(cmlc:actc,ba:egfp)* fish was 1·8 fold that of the WT fish although this difference was not quite statistically different ($P > 0{\cdot}05$). There was no significant difference in the metabolic rate of the *tg(cmlc:egfp)* fish and that of the *tg(cmlc:actc,ba:egfp)* fish or WT fish at U_{crit}. In addition, as would be predicted, the metabolic rate at U_{crit} for the three experimental groups was higher than that measured in the same fish at 50% U_{crit} and the acclimation speed (1 L_B s^{-1}) ($P < 0{\cdot}05$). For the WT fish the metabolic rate was 1·53 and 1·84 fold higher at U_{crit} compared with 50% U_{crit} and the acclimation speed [$P < 0{\cdot}05$; Fig. 2(b)]. For the *tg(cmlc:egfp)* fish the metabolic rate was 2·2 and 2·5 fold higher at U_{crit} compared with 50% U_{crit} and the acclimation speed [$P < 0{\cdot}05$; Fig. 2(b)]. Finally, the metabolic rate of the *tg(cmlc:actc,ba:egfp)* fish was 3·6 and 4·1 fold higher at U_{crit} than at 50% U_{crit} ($P < 0{\cdot}05$) and the acclimation speed. There was no significant difference in the metabolic rate of all groups when swimming at the acclimation speed or at 50% U_{crit}.

COST OF TRANSPORT

At U_{crit}, the C_T for the *tg(cmlc:actc,ba:egfp)* fish was 2·1 fold higher than that of the WT fish ($P < 0{\cdot}01$). There was, however, no significant difference in the C_T between

TABLE I. Mean ± S.E. morphological characteristics and mortality rates of adult male *Danio rerio*

	n	Wet body mass (g)	Total length (cm)	Condition factor (*K*)	Mortality (%)
Wild-type	10	$0{\cdot}76 \pm 0.08^{a}$	$4{\cdot}28 \pm 0.15^{a}$	$0{\cdot}95 \pm 0.06^{a}$	11·76
tg(cmlc:egfp)	9	$0{\cdot}44 \pm 0.03^{b}$	$3{\cdot}43 \pm 0.09^{b}$	$1{\cdot}09 \pm 0.05^{ab}$	12·90
tg(cmlc:actc,ba:egfp)	5	$0{\cdot}68 \pm 0.04^{ab}$	$3{\cdot}78 \pm 0.09^{b}$	$1{\cdot}26 \pm 0.04^{b}$	50·00

n, is the number of different fish characterized per treatment.

Values in the same column indicated by different superscripts are significantly different ($P < 0{\cdot}05$) Per cent mortality was calculated from the total number of fish that were being housed within the facility during the 7 month duration of the experiments.

the *tg(cmlc:egfp)* fish and that of the *tg(cmlc:actc,ba:egfp)* fish at U_{crit}. The C_T of the *tg(cmlc:egfp)* fish was 1·45 fold that of the WT at U_{crit}. This difference, however, was not statistically significant ($P > 0{\cdot}05$). The C_T between all groups was similar when swimming at the acclimation speed and 50% U_{crit}. As would be predicted the C_T in all fish decreased as the rate of swimming increased. For the WT fish the C_T at U_{crit} was approximately 64% and 22% that of when the fish were swimming at 50% U_{crit} and the acclimation speed, respectively [$P < 0{\cdot}05$; Fig. 2(c)]. Similarly, the C_T for the *tg(cmlc:egfp)* fish at U_{crit} was approximately 99% and 28% that of when the fish were swimming at 50% U_{crit} and the acclimation speed, respectively [$P < 0{\cdot}05$; Fig. 2(c)]. Finally, the C_T for the *tg(cmlc:actc,ba:egfp)* fish at U_{crit} was approximately 181% and 52% that of when the fish were swimming at 50% U_{crit} and the acclimation speed, respectively [$P < 0{\cdot}05$; Fig. 2(c)].

FISH MORTALITY, MORPHOLOGY AND CONDITION FACTOR

Records were maintained over the 7 month period of this experiment to track mortalities in the different *D. rerio* lines post hatch. These data demonstrated that the rate of mortality was higher in the *tg(cmlc:actc,ba:egfp)* line compared with the WT *D. rerio* and the *tg(cmlc:egfp)* line (Table I). The mean wet mass of the WT fish was 72% greater than that of the *tg(cmlc:egfp)* fish ($P < 0{\cdot}05$; Table I). There was no significant difference in mean wet mass of the *tg(cmlc:actc,ba:egfp)* fish and that of the *tg(cmlc:egfp)* fish and WT fish. The mean length of the WT fish was 25% longer than that of the *tg(cmlc:egfp)* fish ($P < 0{\cdot}05$) and 13% longer compared with the *tg(cmlc:actc,ba:egfp)* fish ($P < 0{\cdot}05$). There was no significant difference in the length of the *tg(cmlc:egfp)* fish and *tg(cmlc:actc,ba:egfp)* fish. The condition factor of the *tg(cmlc:actc,ba:egfp)* fish was 31% higher compared to that of the WT fish ($P < 0{\cdot}01$). A higher condition factor indicates that the *tg(cmlc:actc,ba:egfp)* fish were in better body condition than the WT fish, potentially suggesting that the differences found in metabolic rate and cost of transport would be larger between the two groups at similar values of the condition factor (*K*), where $K = 100 m L_B^{-3}$ and is a relative index of fish health. There was no significant difference in the condition factor of *tg(cmlc:egfp)* fish and that of the *tg(cmlc:actc,ba:egfp)* fish and WT fish.

DISCUSSION

The goal of this study was to determine if the expression of eGFP and human ACTC transgenes influence the biology of the *D. rerio*. The lower heart rate of the *tg(cmlc:-actc,ba:egfp)* embryos compared with that of the WT embryos and the higher rate of oxygen consumption and C_T at U_{crit}, in the adult *tg(cmlc:-actc,ba:egfp)* fish indicates that there is an effect of the manipulation.

ANIMAL HEALTH

Fish that were expressing both eGFP and human ACTC had the highest rate of mortality as well as the lowest heart rate. The different fish lines used in this experiment, while kept in separate tanks, were maintained under identical conditions, including density and water source. This indicates that the expression of eGFP and ACTC had affected the health of the fish. In the developing embryo, the pressure generation by the heart is essential to the development of the cardiovascular system as well as the transport of metabolites and oxygen at later developmental stages (Miller *et al*., 2008; Miller *et al*., 2011). Reduced cardiac function has significant potential to affect the long-term survival of the animal (Miller *et al*., 2008; Miller *et al*., 2011). There was no significant difference in the heart rates of the *tg(cmlc:egfp)* embryos compared with that of the WT embryos and there was no significant difference in the rates of mortality or in measures of aerobic performance in the adult fish of these two groups. Finally, the embryonic heart rates reported in this study for the WT *D. rerio* are within 10% of rates previously reported for the same developmental stage (Ho *et al*., 2007; Denvir *et al*., 2008).

METABOLIC RATE, COST OF TRANSPORT AND CRITICAL SWIMMING SPEED

The approximately two-fold higher metabolic rate and C_T of the *tg(cmlc:actc,ba:egfp)* fish at U_{crit} compared with the WT fish suggests that the expression of eGFP throughout the whole organism and ACTC in the heart has a metabolic consequence at high levels of aerobic exercise. As these effects were only seen at high levels of activity, the cause of the additional metabolic cost is not likely to be maintenance processes, such as protein synthesis. While the experimental design does not make it possible to state explicitly which of the two manipulations is responsible for the functional difference, there are a number of plausible explanations for this result. The first is that the higher metabolic rate and C_T at U_{crit} in the *tg(cmlc:actc,ba:egfp)* fish is due to a change in the contractility of either the aerobic swimming muscle or heart, caused by changes to the integrity or organization of the contractile element. eGFP is expressed throughout the animal except the heart, while ACTC is only expressed in the heart. As the heart makes up less than 0·1% of a fishes body mass (Klaiman *et al*., 2011) it is unlikely that a change in its metabolic rate could cause a significant increase in whole animal metabolic rate. This suggests then it is changes to the function of the swimming muscles. In fish, sustained swimming is powered by the aerobic red muscle fibres and these are fuelled by the oxidation of lipids and carbohydrates (Weber *et al*., 1996). Work by Agbulut *et al*. (2007) suggests that the expression of eGFP in rat *Rattus norvegicus* cardiac myocytes can affect the orientation of the myosin heads bound to the actin filaments and impair contractile function. Such an effect could have a consequence on the ATP utilization by the myocytes by altering the kinetics of cross-bridge formation.

For example, a decrease in the strength of cross-bridge attachment would decrease the force generated per unit muscle and require an increase in the amount of muscle activated to generate a similar amount of force in the swimming fish. The end result would be a higher rate of acto-myosin ATPase activity and therefore greater ATP utilization by the animal.

A change in the contractile function of the swimming muscle has the potential to induce the fish to alter their swimming gait and as a result, affect oxygen utilization by the animal. Such a response could also explain the results of the current study. In fishes, the swimming gait can be thought of as the pattern of motion used by the animal to generate locomotor force against the surrounding water. The force is generated by the sides of the animal and the fins, including the tail (Altringham & Ellerby, 1999). Fishes alter their gait to match swimming speed and environmental conditions so as to maximize muscle power (Peake & Farrell, 2004). To swim at a faster rate, fishes will activate more muscle mass and this will translate into a shift in swimming gait as more or different muscle is activated (Peake & Farrell, 2004). This response acts to increase the C_T in part as the resistance to the animal moving through the water is increased (Svendsen *et al*., 2010). If the expression of eGFP in the skeletal muscle affects muscle function thereby requiring an increase in muscle activation than this could have a similar effect.

Another potential explanation for the increase in C_T at U_{crit} in the *tg(cmlc:actc,ba:egfp)* fish is an increased reliance on lipids to fuel aerobic ATP production at high levels of exercise (U_{crit}). This hypothesis is proposed because work by Li *et al*. (2013) using mass spectroscopy for metabolomic analyses suggested that the expression of eGFP in transgenic mice caused changes in the maintenance of glucose delivery to the tissues as indicated by reduced levels of glycogen and glucose in the liver of eGFP mice. In addition, experiments by Choi & Weber (2016) on rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) have demonstrated that the C_T at high levels of aerobic exercise decreased when the fish were supplied with exogenous glucose. But that the U_{crit} was not affected. These authors suggested that increased usage of glucose and a decreased reliance on lipids, for ATP production decreased oxygen utilization by the fish. Such a change in metabolic fuel source would reduce oxygen requirements by 15 to 30% while generating the same amount of ATP (Welch *et al*., 2007; Schippers *et al*., 2012). Therefore, if eGFP expression in the *D. rerio* is reducing glycogen delivery to the tissue, this could increase reliance on lipid stores and therefore increase oxygen utilization. In the current study, the *tg(cmlc:egfp)* fish showed a trend towards an increase in metabolic rate at U_{crit}; however, eGFP expression was heart-specific. It is possible that there is a greater effect of eGFP expression on the metabolic rate in the *tg(cmlc:actc,ba:egfp)* fish, where it is expressed throughout the body.

The metabolic rates reported in this study for adult *D. rerio* at slower speeds (0–4 L_B s^{-1}) are similar to those reported in previous studies (Masse *et al*., 2013; Vergauwen *et al*., 2013; Gerger *et al*., 2015). At higher speeds, however, there is greater variability between studies, especially when comparing the metabolic rates associated with U_{crit}. This variation can be attributed to differences in the acclimation protocols used, as well as in swim-tunnel design. As a result, there is variation in the U_{crit} values determined between studies. For example the U_{crit} for WT in this study was 7·5 L_B s^{-1}, while that reported by Masse *et al*. (2013) was *c*. 9·5 L_B s^{-1}. As the metabolic rates, however, and swimming capacity of all fish in the current study were measured on the same equipment, however, the comparisons between the treatment groups are valid.

It is interesting to note that there was no statistical difference in metabolic rate measured at the acclimation speed of 1 L_B s^{-1} and at 50% U_{crit} (approximately 4 L_B s^{-1}) for any of the three groups. This probably reflects an increase in swimming efficiency *via* changes in behaviour and swimming gait of the fish as water speed increases. In addition, as the metabolic rate at U_{crit} can be taken as an indicator of the maximal rate of oxygen utilization (M_{O2max}) the results of the current study could be interpreted to indicate that the M_{O2max} of the *tg(cmlc:actc,ba:egfp)* fish is significantly greater than that of the WT fish. Such a response could be due to increases in haematocrit, angiogenesis in the swim muscles or increased cardiac function. As it was not possible to sacrifice the fish, these characteristics were not measured. Examining this response and identifying its trigger, should be the focus of future work.

In conclusion, while *D. rerio* have significant potential as a model species to examine the physiological consequence of genetic mutations associated with cardiomyopathies, it is clear that care needs to be taken to assess the consequence of all genetic manipulations. The almost two-fold higher metabolic rate at U_{crit} of the *tg(cmlc:actc,ba:egfp)* fish suggests that the swimming efficiency of the animal is affected. Such an effect has potential consequences on the long-term health and survival of the animal through increased energy requirements. As heart disease can be caused by mitochondrial dysfunction leading to changes in ATP production (Lesnefsky *et al*., *b, c*), manipulations that alter metabolic function have the potential to affect the results of studies of cardiomyopathies caused by genetic mutations. The results of this study should therefore be considered in future experiments that use eGFP as a cellular marker of genetic manipulation or that induce the expression of novel protein isoforms using a transgenic approach.

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Supporting Information

Supporting Information may be found in the online version of this paper:

FIG. S1. Wet mass-specific oxygen consumption rates of trial adult wild-type zebrafish (n = 5) at 28°C, acclimating to a swimming speed of 1·0 body lengths/second over 2 h.

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