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Novel insights into cardiac remodelling revealed by proteomic analysis of the trout heart during exercise training

Laura A. Dindia [1], Sarah L. Alderman [1], Todd E. Gillis *

Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada

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ABSTRACT

The changes in the cardiac proteome of rainbow trout (*Oncorhynchus mykiss*) were quantified during the early phases (4, 7, and 14 d) of a typical exercise-training regime to provide a comprehensive overview of the cellular changes responsible for developing a trained heart phenotype. Enhanced somatic growth during the 14 d experiment was paralleled by cardiac growth to maintain relative ventricular mass. This was reflected in the cardiac proteome by the increased abundance of contractile proteins and cellular integrity proteins as early as Day 4, including a pronounced and sustained increase in blood vessel epicardial substance – an intercellular adhesion protein expressed in the vertebrate heart. An unexpected finding was that proteins involved in energy pathways, including glycolysis, β-oxidation, the TCA cycle, and the electron transport chain, were generally present at lower levels relative to Day 0 levels, suggesting a reduced investment in the maintenance of energy production pathways. However, as the fish demonstrated somatic and cardiac growth during the exercise-training program, this change did not appear to influence cardiac function. The in-depth analysis of temporal changes in the cardiac proteome of trout during the early stages of exercise training reveals novel insights into cardiac remodelling in an important model species.

Biological significance: Rainbow trout hearts have a remarkable ability for molecular, structural, and functional plasticity, and the inherent athleticism of these fish makes them ideal models for studies in comparative exercise physiology. Indeed, several decades of research using exercise-trained trout has shown both conserved and unique aspects of cardiac plasticity induced by a sustained increase in the workload of the heart. Despite a strong appreciation for the outcome of exercise training, however, the temporal events that generate this phenotype are not known. This study interrogates the early stages of exercise training using in-depth proteomic analysis to understand the molecular pathways of cardiac remodelling. Two major and novel findings emerge: (1) structural remodelling is initiated very early in training, as evidenced by a general increase in proteins associated with muscle contraction and integrity at Day 4, and (2) the abundance of proteins directly involved in energy production are decreased during 14 d of exercise training, which contrasts the general acceptance of an exercise-induced increase in aerobic capacity of muscle, and suggests that regulation of energy pathways occurs at a different biological level than protein abundance.

1. Introduction

Exercise training induces a suite of physiological changes to improve oxygen convection, energy utilization, and metabolic waste removal, resulting in improved aerobic performance. Among these changes, cardiac plasticity is critical given the central role of heart function during exercise and recovery. The fish heart is a useful model for studies of exercise-induced cardiac plasticity in vertebrates for three reasons. First, fish hearts have a remarkable ability to remodel in response to physiological stressors [1], including thermal acclimation [2–5], anaemia [6], sexual maturation [7–9] and exercise [10–12]. Second, key differences in how the fish heart responds to training compared to the mammalian heart, including myocyte hyperplasia [12,13] and increased mitochondrial activity [12,14], suggests that novel pathways are activated in response to the increase in workload brought on by exercise. A deeper understanding of these phylogenetic differences could lead to important advances in the treatment of various heart maladies. Lastly, exercise can improve disease resistance [15] and somatic growth in fish [16,17], which are advantageous outcomes for aquaculture. This presents a direct application for knowledge gained in exercise studies on commercially relevant fish species [18]. Indeed, the high commercial value of salmonids like the rainbow trout (*Oncorhynchus mykiss*),

* Corresponding author at: Dept. Integrative Biology, University of Guelph, 50 Stone Rd E., Guelph, ON, N1G 2W1, Canada.
E-mail address: tgillis@uoguelph.ca (T.E. Gillis).
URL: http://comparativephys.ca/gillislab/ (T.E. Gillis).
[1] Author contributed equally.

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combined with their natural athleticism, makes this group of teleosts a model of choice in exercise studies on fish.

Cardiac function and morphology is directly linked to swimming performance in fish [19]. Following at least 4 wk of training at sustainable swimming speeds (≤2 body lengths per second, BL s^{-1}), a number of cellular, biochemical, and functional properties of the heart are modified. The metabolic capacity of the heart improves, as demonstrated by increases in the activity of oxidative enzymes [14], and in mitochondrial surface area and mitochondrial DNA content [12]. Maximum power output of the heart increases [14], as does maximum oxygen consumption [11]. Physiological hypertrophy is induced by exercise training, including increased expression of myocyte growth factors (ex. myogenin) and cell proliferating genes (ex. proliferating cell nuclear antigen) [12,20], and a modest increase in relative ventricular mass often occurs [10–12], but not always [14]. However, differences in training intensity and duration can influence the cardiac phenotype that emerges from a given study, which may explain why improved performance in a standardized swimming test (U_{crit}) is sometimes [21,22] but not always observed [11,14]. In mammals, too, it is well-established that while moderate exercise is cardioprotective [23], prolonged and high intensity activity can lead to paraphysiological adaptations resembling features of cardiomyopathy [24,25].

Building on the well-characterized cardiac phenotype that results from aerobic training in fish, the goal of the present study was to interrogate the underlying mechanisms that drive exercise-induced cardiac plasticity in fish by quantifying the temporal changes in the cardiac proteome of rainbow trout during the early phases of exercise training. This was accomplished using isobaric tag for relative and absolute quantitation (iTRAQ) paired with mass spectrometry to quantify each identified protein. This approach enabled a global perspective of the temporally sensitive cellular changes that produce a trained heart phenotype.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (*O. mykiss*) of mixed sex and uniform size (n = 27; 21.6 ± 0.31 cm; 118.9 ± 5.7 g) were obtained from Alma Aquaculture Research Station (Elora, Ontario) and acclimated for 4 wk in the Hagen Aqualab (University of Guelph) in 2000 L tanks held at 11 °C with a 12 h light/12 h dark photoperiod. Fish were fed Profishent fish feed (Martin Mills Inc., Elmira ON), *ad libitum* once daily during the acclimation and experimentation periods. All protocols were approved by the University of Guelph Animal Care Committee (Protocol #2710), as per the guidelines of the Canadian Council for Animal Care.

2.2. Exercise training

A circular raceway was constructed by placing a 1 m diameter plastic pipe vertically in the center of the tank and then injecting water tangentially to create a current. A flow meter was used throughout the experiment to measure water velocity at multiple locations. Exercise training was initiated by gradually increasing water velocity from 0 cm s^{-1} to 40 cm s^{-1} over a 24 h period (approximately 2.0 BL s^{-1} on Day 1, and 1.7 BL s^{-1} on Day 14, based on average fork length). This velocity was chosen to estimate 60% U_{crit} for rainbow trout of similar size [26], and reflects previous studies that observed changes in relative ventricular mass [10] and cardiac function [14] in trout swum at similar speeds for 4 wk. Prior to increasing water velocity (Day 0), and then on days 4, 7, and 14, a sub-sample of trout were euthanized in buffered MS-222 (50 mg l^{-1}; n = 6–7 per time point). Mass and fork length were recorded and used to calculate condition (K) factor using the formula:

$$K = 10^5 \times \text{mass(g)}/\text{length(mm)}^5$$

Blood was collected by free-flow from caudal vessels, allowed to clot for 1 h at room temperature, cleared by centrifugation, and then the serum was isolated and frozen. The ventricle was removed and rinsed twice with saline buffer to clear luminal blood, then snap frozen on dry ice. Gender was assigned based on the colour of the developing gonads, with ovaries having a distinct orange hue relative to the pale pink testes; however, all fish were sexually immature.

2.3. Glucose and lactate assays

Serum glucose and lactate concentrations were determined spectrophotometrically by fitting absorbance values against standard curves generated from known dilutions of glucose or lactate, respectively. Serum glucose was quantified in duplicate 200 μl reactions (0.2 M Tris, 5 mM NAD, 2 mM $MgSO_4$, 5 mM ATP, 0.08 U glucose-6 phosphate dehydrogenase Sigma-Aldrich CAS 9001-40-5; pH 7.4) by measuring the change in absorbance (340 nm) after incubating for 45 min at room temperature in the presence of 0.1 U hexokinase (Sigma-Aldrich, CAS 9001-51-8). Serum lactate was quantified in duplicate 200 μl reactions (0.2 M hydrazine sulfate, 0.5 mM NAD; pH 9.5) by measuring the change in absorbance (340 nm) after incubating for 30 min at room temperature in the presence of 5 U lactate dehydrogenase (Sigma-Aldrich, CAS 9001-60-9).

2.4. iTRAQ labelling

The ventricles of 2 male and 2 female fish per time point (except Day 7 which had 2 males and 1 female) were manually pulverized with a mortar and pestle, then homogenized in SDS buffer (4% w/v SDS, 100 mM HEPES, 0.1 M DTT, pH 7.6) containing 1× protease inhibitor (Roche, Mississauga, ON). The crude homogenate was clarified by centrifugation at 16,000 g for 10 min. Proteins from the supernatant were extracted using the Calbiochem Protein Extraction Kit (EMD Millipore, Billerica, MA), as instructed by the manufacturer. The protein pellet was dissolved in HEPES buffer (1 M HEPES, 8 M urea, 2 M thiourea, 4% CHAPS *w*/v; pH 8.5) and protein concentration determined using the Pierce BCA Protein Assay Kit (Thermo-Fisher, Whitby, ON). For each sample, 200 μg of protein was transferred to an Amicon® Ultra-0.5 centrifugation filter device (10 K nominal molecular weight limit), in which samples were washed three times with UA buffer (8 M urea in 0.1 M HEPES, pH 8.5) before being incubated for 30 min in the dark with UA buffer containing 0.05 M of iodoacetamide (IAA, Sigma-Aldrich, Oakville, ON) and washing three times with 0.5 M of triethylammonium bicarbonate (TEAB, Sigma). Sequence-modified trypsin (Thermo-Fisher) was dissolved in 0.5 M TEAB and was added to each sample at a 1:50 enzyme to protein ratio. Samples were trypsin digested overnight (approximately 18 h) at 37 °C.

Digested peptides from the 4 biological replicates per time point were labelled using 2 8-plex iTRAQ kits (Thermo-Fisher), as outlined in the manufacturer's protocol. One Day 0 sample was labelled in duplicate reactions for inclusion as a standardization control across the 2 plexes. All experimental groups were represented within each 8-plex. Isobaric tags were alternated between experimental groups to avoid the unlikely potential for labelling bias between tags [27]. Following peptide labelling, samples within each 8-plex were pooled and purified through a C18 column (Sigma). Labelled peptides were eluted with 70% acetonitrile and 0.1% formic acid.

2.5. Mass spectrometry

Mass spectrometry was carried out at SickKids Proteomics Analysis Robotics & Chemical Biology Centre (SPARC BioCentre, Toronto, ON). Peptides were loaded onto a 50 cm × 75 μm ID column with RSLC 2 um C18 resin (EASY-Spray, Thermo-Fisher) with an integrated emitter. The Easy-Spray nLC 1000 chromatography system (Thermo-Fisher) was used to elute peptides onto a Q-Exactive hybrid mass spectrometer

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(Thermo-Fisher) using a solvent gradient (0 to 35% acetonitrile in 0.1% formic acid) over 4 h. The mass spectrometer was operated in the data dependent mode with 1 MS followed by 10 MS/MS spectra. Resolution of MS scans were either 70,000 (MS) or 17,500 (MS/MS) FWHM, with a target of 1 × 106 ions and maximum scan time of 120 ms. A relative collision energy of 27% was used for MS/MS. First mass was fixed at 80 Da with a dynamic exclusion of 15 s for MS/MS scans. Raw data files were acquired with XCalibur 2.2 and processed with Proteome Discover 1.4 (Thermo-Fisher).

2.6. Western blotting

The remaining frozen ventricles that were not processed for iTRAQ (n = 2–4 per time point) were pulverized and briefly sonicated in ice-cold buffer (50 mM Tris-HCl, pH 7.5, containing 1× protease inhibitor (Roche)), then cleared by centrifugation. The protein concentration of the supernatant was determined using a BCA assay (Thermo-Fisher), and then samples were diluted in Laemmli's buffer (60 mM Tris-HCl, 10% glycerol, 2% SDS, 0.01% bromophenol blue, 5% 2-mercaptoethanol, pH 6.8) and boiled (95 °C, 5 min). Approximately 20 μg total protein was loaded per lane of a 12% polyacrylamide gel and separated by SDS-PAGE, with all time points represented on each gel. Separated proteins were transferred onto a PVDF membrane (30 V for 16 h), and then stained for total protein using SYPRO Ruby Blot Stain (Invitrogen) as per manufacturer's instructions. Blots were blocked in 5% skim milk in tris-buffered saline with 0.1% Tween-20 (TBST) then incubated overnight at 4 °C in a custom-made rabbit polyclonal antibody against salmonid cardiac troponin I (cTnI; 1:500 in blocking buffer; Cedarlane Laboratories, Burlington, ON). Immunodetection was completed by incubating blots for 1 h at room temperature in goat anti-rabbit IgG-HRP (1:3000 in blocking buffer; Santa Cruz Biotechnologies, Dallas, TX) and visualizing with enhanced chemiluminescence on a ChemiDoc MP (BioRad). Densitometry was performed using Image Lab 5.2 (Bio Rad) and cTnI abundance was standardized against total protein and normalized to the loading control.

2.7. Data analysis

Statistical differences in fish metrics, serum metabolite concentrations, and cTnI abundance (densitometry) were determined using a one-way analysis of variance (ANOVA; $p < 0.05$) followed by a Tukey HSD test for pairwise comparisons where differences were detected.

Identification and quantification of cardiac proteins was performed by PEAKS Studio 7 (Bioinformatics Solutions Inc., Waterloo, ON, Canada). The mass spectra were searched against the ray-finned fishes (*Actinopterygii*) NCBI non-redundant protein database and Uniprot/Swissprot database (taxonomy not selected) downloaded on June 16, 2015. Carbamidomethylation was set as a variable modification and iTRAQ as a fixed modification on N-termini and lysine residues. Search parameters allowed up to two missed trypsin cleavages, 10.0 ppm parent mass error tolerance and 0.02 Da fragment mass error tolerance. A high confidence in the peptide-protein identifications was assured by using a FDR cut-off of 1% and requiring a protein identification score (−10logP) of ≥20 [28] and at least one unique peptide for protein identification [29,30]. More than 99% of proteins had a protein identification score > 30 (equivalent P-value = 0.001), indicating these scores have <0.1% chance of being a random match, and all proteins included in the analysis were identified on both 8-plexes. An identical sample was included on each 8-plex to standardize abundance values between the two plexes. Once standardized, the two 8-plexes were combined for analysis, and only proteins that were quantified in all samples from both 8-plexes were considered for analysis. Statistical analysis of differentially abundant proteins was conducted using Linear Models for Microarray Analysis (LIMMA) implemented as a Bioconductor package in the R statistical interface [31]. Prior to statistical analysis, abundance values were globally normalized using variance stabilization in the R-based package 'VSN' [32]. Protein quantification using iTRAQ tends to underestimate fold changes compared to other approaches, therefore uncorrected P-values < 0.05 were used to determine significant changes [33]. Differentially abundant proteins that were classified as unnamed proteins from the NCBI database were identified using Blastp, and then assigned the corresponding human ortholog protein accession ID. Differentially abundant proteins were omitted from pathway analysis if a human ortholog could not be confidently assigned (9 proteins), or if they were not part of the true cardiac proteome (6 blood proteins, and residual trypsin from digestion step). Protein ontology of the whole ventricle proteome was conducted using Blast2GO [34]. To characterize the pathways involved in exercise-induced cardiac remodelling the Search Tool for the Retrieval of INteracting Genes/proteins (STRING) was used to create functional networks of the differentially abundant proteins for each day during the training regimen. Heat maps were generated from median z-scores of the differentially abundant proteins using complete-link hierarchical clustering in the open source InfernoRDN statistical software for proteomic analysis [35].

3. Results

3.1. Organismal response to exercise

Average fish mass increased by 50% during the 14 d exercise period ($p < 0.05$), but there was no significant change in fork length (Table 1). The *K* factor remained relatively stable throughout the experiment, with no time point being significantly different from Day 0, but Day 14 was significantly greater than Day 4 ($p < 0.01$). A significant difference in ventricle mass was detected among time points by ANOVA, reflecting an increase in mass with time, but post-hoc analysis failed to detect pairwise differences (Table 1). There was no difference in relative ventricular mass (Table 1). There was no effect of exercise on serum glucose levels, while the serum lactate levels were higher at Day 7 compared to Days 0, 4, and 14 (Table 1).

3.2. General summary of trout cardiac proteome

The mass spectra from the 2 iTRAQ 8-plexes were searched against the NCBI-nr (*Actinopterygii*) and Uniprot/Swissprot (all taxonomy) databases, returning 736 and 615 protein identifications, respectively (see Supplementary data Table 1 in [36]). The assignment of peptide ratios based on isobaric labelling was 90%, which is similar to previously reported labelling efficiencies for iTRAQ [37]. Protein ontology from Blast2Go analysis categorized proteins into cell compartment, biological

Table 1
Body mass, fork length, condition factor (*K*), ventricle mass, relative ventricular mass (RVM), and serum concentrations of glucose and lactate during 14 days of continuous exercise training. An * in the main headings indicates that a significant difference in that variable was detected using a one-way ANOVA, and pairwise differences were determined with a Tukey test and are denoted by letters ($p < 0.05$; $n = 6–7$). Different letters indicate a significant difference between days.

	Mass* (g)	Length (cm)	*K* Factor*	Ventricle* (mg)	RVM ($\times 10\ e^{-3}$)	Glucose (mM)	Lactate* (mM)
Day 0	102.3 ± 3.5^{a}	20.7 ± 0.3	1.16 ± 0.06^{ab}	173 ± 6^{a}	1.7 ± 0.09	2.2 ± 0.1	3.3 ± 0.5^{a}
Day 4	99.1 ± 5.7^{a}	21.1 ± 0.6	1.05 ± 0.04^{a}	161 ± 13^{a}	1.6 ± 0.09	2.2 ± 0.2	4.2 ± 0.6^{a}
Day 7	124.8 ± 11.1^{ab}	21.9 ± 0.7	1.16 ± 0.02^{ab}	200 ± 18^{a}	1.6 ± 0.09	2.1 ± 0.1	5.5 ± 0.4^{b}
Day 14	147.1 ± 11.6^{b}	22.4 ± 0.7	1.30 ± 0.03^{b}	221 ± 19^{a}	1.5 ± 0.04	2.1 ± 0.1	3.5 ± 0.3^{a}

RVM, relative ventricular mass calculated by dividing ventricle mass by body mass for individual fish.

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process and molecular function, and included 11–17 subcategories within each group (Fig. 1). Intracellular proteins were the most populated subcategory within cell compartment. Cellular and metabolic processes were the top subcategories within biological processes. Finally, binding and catalytic activity dominated the molecular function category.

3.3. Cardiac proteomic response to prolonged exercise

A heat map was constructed to visualize the hierarchical clustering of protein abundances across time points (Fig. 2). The heat map confirms that the cardiac proteomes of biological replicates (individual fish) are highly similar within time points, and that exercise training exerts temporally-specific changes to this proteome. The greatest proteomic response occurred at Day 4 and is distinct from the responses at Days 7 and 14 (Fig. 2). In total, the abundance of 108 proteins were significantly altered in response to exercise compared to non-exercised trout, with 68, 33, and 49 proteins differentially abundant at Days 4, 7, and 14, respectively (Fig. 3; also see Supplementary data Table 2 in [36]; LIMMA, $p < 0.05$). There were few similarities between time points in the specific proteins that were altered, with only 7 proteins being significantly different from controls at Day 4, 7, and 14 (Fig. 3; Table 2). These temporally-independent responses to exercise included increases in calponin-1 (average fold change from controls (FC) = 1.41), blood vessel epicardial substance (BVES; FC = 1.35), and vinculin-like isoform X2 (FC = 1.26), and decreases in ATP synthase O subunit mitochondrial precursor (FC = 0.71), carnitine O-palmitoyltransferase (CPT1B; FC = 0.65), protein quaking-A (FC = 0.80) and cytochrome b-c1 complex (FC = 0.83).

Functional networks of the differentially abundant proteins for each day during the training regimen were generated to begin interpreting the exercise-induced proteomic response. Networks generated from differentially abundant proteins on each sampling day had a significantly higher number of interacting proteins than expected by chance. The highest clustering coefficient occurred on Day 4 (0.77; Fig. 4A), while Days 7 and 14 had clustering coefficients of 0.62 and 0.69, respectively (Fig. 4B–C). Three main functional categories emerged within the networks: muscle contraction, metabolic pathways, and structural integrity. Each of these is elaborated on in more detail below.

3.4. Effects of exercise on contractile proteins

Common among the network analyses for Days 4, 7, and 14 were protein clusters associated with muscle contraction. The increased abundance of myomesin 1, myosin heavy chain, and cTnI, as well as a decrease in the anti-hypertrophic and inotropic protein glutaredoxin 3 at Day 4 of exercise training support a hypertrophic remodelling

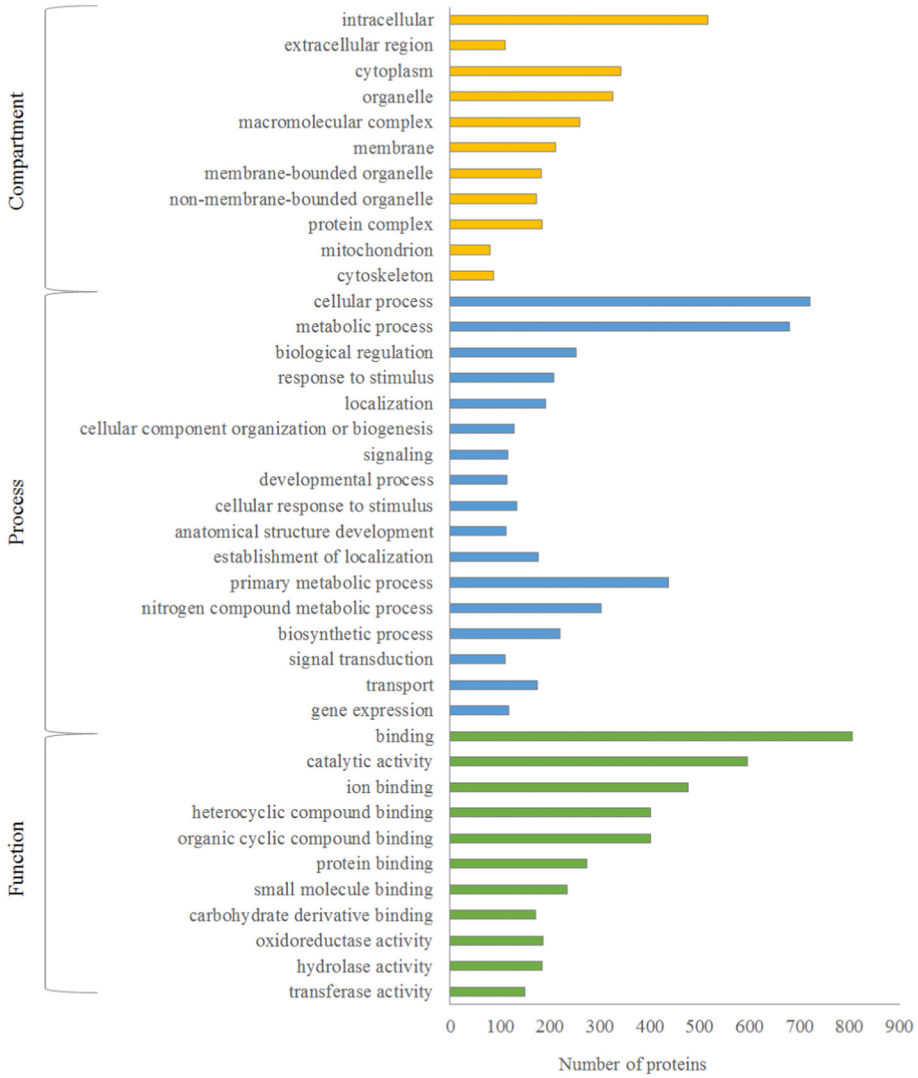

Fig. 1. GO distribution of the 1351 proteins quantified in the non-exercised (Day 0) trout heart by searching mass spectra data against the NCBI nr and Uniprot/Swiss databases.

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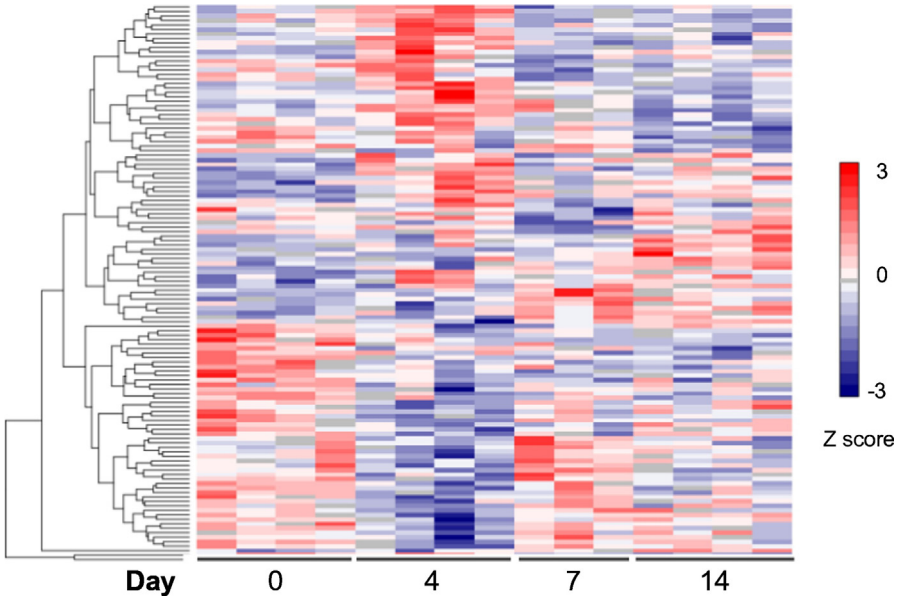

Fig. 2. Heat map of hierarchical clustering of differentially abundant cardiac proteins in the ventricles of individual fish at Day 0, Day 4, Day 7, and Day 14 of exercise training. Colours represent relative abundance levels for each protein across time points based on z-scores.

response (Table 2). Many of these proteins returned to Day 0 levels after Day 4, except for myosin 8 which increased at Day 7, as well as tropomyosin alpha-1 and titin-like which were both elevated at Day 14 (Table 2). Western blotting was used to validate the effect of exercise on cTnI abundance and confirmed a significant 2-fold increase at Day 4 and a return to Day 0 levels by Day 7 (Fig. 5; $n = 2–4$, $p < 0.05$). In further support of a general hypertrophic response to exercise, there was a sustained increase in the thin filament-associated regulatory protein, calponin-1, and a sustained decrease in the translational repressor, protein quaking-A, in the hearts of all exercised fish relative to controls (Table 2), as well as transient increases in several transcriptional and translational regulators (see Supplementary Table 2 in [36]).

3.5. Effects of exercise on metabolic proteins

Three energy metabolism pathways were distinguished in the network analysis of differentially abundant proteins, namely carbohydrate and amino acid metabolism, lipid metabolism, and the electron transport chain. These networks were densely populated at Day 4 (Fig. 4A), and less so at Day 7 (Fig. 4B) and Day 14 (Fig. 4C). One of the largest effects of prolonged exercise on metabolic proteins was on those associated with glucose metabolism, including proteins involved in glycolysis and gluconeogenesis (Table 2). More specifically, there were transient increases in several proteins involved in glycolysis (triosephosphate isomerase, phosphoglycerate mutase 1 and 2, enolase, and gamma-enolase). However, there were also transient decreases in proteins involved in glycolysis and gluconeogenesis (glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase A and A–B chain, glucose-6-phosphate isomerase, and pyruvate kinase). With only 2 exceptions (triosephosphate isomerase, enolase), the exercise-induced increases in the glycolytic proteins returned to pre-exercise levels by Day 14. In general, the hearts of exercised fish had lower levels of enzymes involved in fatty acid β-oxidation, including a sustained decrease in CPT1B at all time points (Table 2). Similarly, with the exception of NADH dehydrogenase 1 alpha (Day 14), subunits of electron transporters within the electron transport chain (ETC) were negatively impacted by exercise training, including a significant reduction in complex II (cyctochrome b-c1 complex) and ATP synthase O subunit at all 3 time points, and a decrease in succinate dehydrogenase at Days 7 and 14 (Table 2). Finally, 2 critical components of the malate-aspartate shuttle were less abundant at Days 4 and 7 compared to Day 0 (cytoplasmic malate dehydrogenase and mitochondrial aspartate aminotransferase).

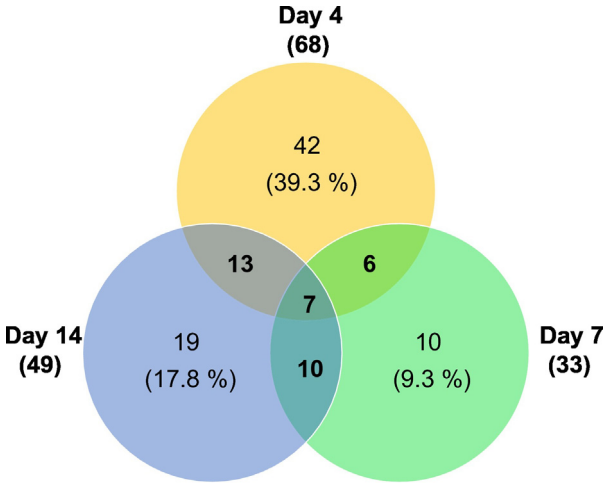

Fig. 3. Venn diagram of the differentially abundant cardiac proteins in exercised fish at Day 4, Day 7 and Day 14 of continuous exercise, relative to non-exercised fish at Day 0.

3.6. Effects of exercise on cell integrity proteins

Exercise training affected protein networks associated with protein binding and stabilization, including changes in the levels of structural proteins associated with the sarcomeres, cytoskeleton, cell-matrix adhesion, extracellular matrix, and cell-cell adhesion. Most of these changes were transient increases. For example several proteins increased at Day 4 and then returned to Day 0 levels, including laminin subunit gamma, lamin-L(III), keratin type II cytoskeletal 8, vinculin isoformX2, and vimentin (Table 2). BVES and vinculin-like isoform X2 were both higher than controls at all time points, and tubulin beta-2 increased over time (Table 2). Other changes to cell integrity proteins included transient decreases in fermitin, and PDZ and LIM domain protein 5 (Table 2).

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Table 2
Selected list of differentially abundant cardiac proteins in the ventricles of rainbow trout exercised for 4, 7, or 14 d, including fold-change from Day 0 (non-exercised) and the associated F-statistic p value. An * indicates a significant fold-change from Day 0. The full list of differentially abundant proteins is available in Supplemental data Table 2 in [36].

Protein	Gene	Accession	Function	Fold-change from Day 0			F
				Day 4	Day 7	Day 14	
Muscle contraction							
Calponin-1	CNN1	CDQ81893	Thin filament regulatory protein	1.524*	1.359*	1.348*	0.004
Cardiac troponin I	TNNI3	NP_001171957	Muscle contraction	1.447*	0.930	1.075	0.006
Glutaredoxin 3	GLRX3	NP_001005950	Inotropic regulator	0.799*	0.970	0.914	0.034
Myomesin-1	MYOM1	NP_001077403	Muscle contraction	1.179*	1.033	0.996	0.018
Myosin heavy chain fast skeletal muscle		Q90339	Muscle contraction	1.219*	1.000	0.962	0.036
Myosin-8	MYH8	NP_001070703	Muscle contraction	1.024	1.442*	1.057	0.001
Titin-like	TTN	XP_011617897	Muscle contraction	1.255*	1.050	1.177*	0.009
Tropomyosin alpha-1 chain isoform X4	TPM1	XP_014031659	Muscle contraction	1.080	1.049	1.439*	0.024
Cell integrity							
Blood vessel epicardial substance	BVES	NP_001001847	Cell adhesion	1.377*	1.336*	1.326*	0.005
Fermitin family homolog 2	FERMT2	NP_001128471	Cell adhesion	0.729*	0.923	0.779*	0.016
Keratin type II cytoskeletal 8	KRT8	NP_956374	Cytoskeleton (muscle)	1.249*	0.999	0.914	0.044
Lamin-L(III)		NP_001081545	Nuclear membrane	1.286*	1.175	0.988	0.027
Laminin subunit gamma-1	LAMC1	NP_775384	Cell adhesion	1.261*	1.111	1.112	0.045
PDZ and LIM domain protein 5	PDLIM5	NP_445778	Cytoskeleton	0.960	0.805*	0.797*	0.031
Tubulin beta-2 chain	TUBB4B	NP_497806	Cytoskeleton	1.000	1.065	1.302*	0.007
Vimentin-1/2	VIM	NP_001080908	Intermediate filament	1.253*	1.047	0.960	0.001
Vinculin isoformX2	VCL	XP_014067877	Focal adhesion	1.205*	0.881	0.91	0.002
Vinculin-like isoformX2		XP_014035770	Focal adhesion	1.349*	1.237*	1.206*	0.005
Metabolism							
Aspartate aminotransferase mitochondrial	GOT2	NP_001016933	Malate-aspartate shuttle	0.847*	1.009	0.882*	0.021
ATP synthase O subunit mitochondrial …	ATP5O	ACO07478	Electron transport chain	0.653*	0.762*	0.705*	0.001
Carnitine O-palmitoyltransferase 2 …	CPT1B	NP_001007448	Lipid metabolism	0.663*	0.683*	0.601*	0.006
Cytochrome b-c1 complex subunit Rieske …	UQCRFS1	CDQ73757	Electron transport chain	0.841*	0.839*	0.809*	0.024
Enolase	ENO3	ABK35075	Glycolysis	1.074	1.170*	1.171*	0.041
Gamma-enolase	ENO2	NP_990207	Glycolysis	1.291*	1.188*	0.981	0.002
Glucose-6-phosphate isomerase glycosomal	GPI	P13377	Glycolysis	0.830	0.534*	0.901	0.046
Glyceraldehyde-3-phosphate dehydrogenase	GAPDHS	BAC06416	Glycolysis	0.731*	0.949	0.882	0.014
L-Lactate dehydrogenase A chain	LDHA	NP_034829	Glycolysis	0.791*	1.119	0.935	0.008
L-Lactate dehydrogenase B-A chain		NP_571322	Glycolysis	0.797*	0.973	0.868	0.019
Malate dehydrogenase cytoplasmic	MDH1	NP_001006694	TCA cycle, malate-asparate shunt	0.652*	0.930	0.709*	0.001
NADH dehydrogenase 1 alpha subcompl …	NDUFA10	CDQ58465	Electron transport chain	0.879	0.962	1.251*	0.017
Triosephosphate isomerase	TPI1	CDQ86074	Glycolysis, lipid metabolism	1.387*	1.153	1.358*	0.022
Other							
Protein quaking-A	QKI	NP_571299	Translational repressor	0.792*	0.764*	0.834*	0.022

4. Discussion

In rainbow trout, there is a strong association between ventricular morphology, cardiac performance, and aerobic swimming capacity [19]. The cardiac phenotype of trout is also highly plastic, and is influenced by both external and internal factors [1]. Therefore, analysis of the cardiac proteome during a well-characterized stimulator of cardiac remodelling – exercise training – is a novel and meaningful approach to understanding plasticity in the trout heart. Moreover, while all studies to date have used long acclimation times (4 wk or more) to study the end cardiac phenotype and its effects on organismal performance, the present study probes the early stages of this remodelling response (4 to 14 d) to gain important insight into the molecular events that drive these functional and morphological changes. Using a comprehensive proteomic approach we find evidence that the ventricle begins remodelling within 4 d of the onset of exercise training, including increases in contractile, adhesion, and ultrastructural proteins. In contrast, metabolic proteins do not keep pace with this hypertrophic response, despite a clear benefit of exercise on somatic and cardiac growth in this study. Here we discuss the temporal response to exercise training in the trout heart with an emphasis on the proteomic changes in functional (muscle contraction), integral (adhesion and ultrastructure), and supporting (metabolism) proteins.

4.1. Organismal response to exercise

The mass gained by the fish over 14 d of exercise training exceeds that predicted for non-exercised fish. Growth in cultured rainbow trout is well-characterized; a 100 g trout will gain 1.6% body weight per day when housed at 11 °C and fed daily to satiation [38]. At this conservative growth rate, the fish in the present study would be expected to weigh 128 g by Day 14 – or put another way, it would take them 25 d to reach the 147 g final weight that we measured after just 14 d. This higher than predicted increase in body mass is supported by previous studies in salmonids showing that exercise at or below 2 BL s^{-1} stimulates somatic growth [15–18,22], and also indicates that the daily caloric intake of fish in this study, fed *ad libitum*, was in excess of a maintenance diet. In addition, the fact that there was no difference in the plasma glucose levels throughout the study suggests that glucose was not limiting during the sustained swimming. Similarly, plasma lactate levels remained relatively low during this study reflecting the moderate intensity of the training regime, although a small rise at Day 7 suggests a temporary increase in reliance on anaerobic metabolism. These organismal effects are important to note, given the observed changes in energy pathways within the heart described below. Finally, there was no difference in the RVM of fish between Day 0 and Day 14, indicating that the heart stayed the same proportion relative to body mass during this period of increased somatic growth. This supports isometric ventricular growth induced by aerobic training in salmonids [10,22], and therefore the exercise-induced changes in the cardiac proteome include this growth response.

4.2. Contractile element

Contraction of striated muscle, powered by the hydrolysis of ATP, is caused by the formation of force generating cross-bridges between the

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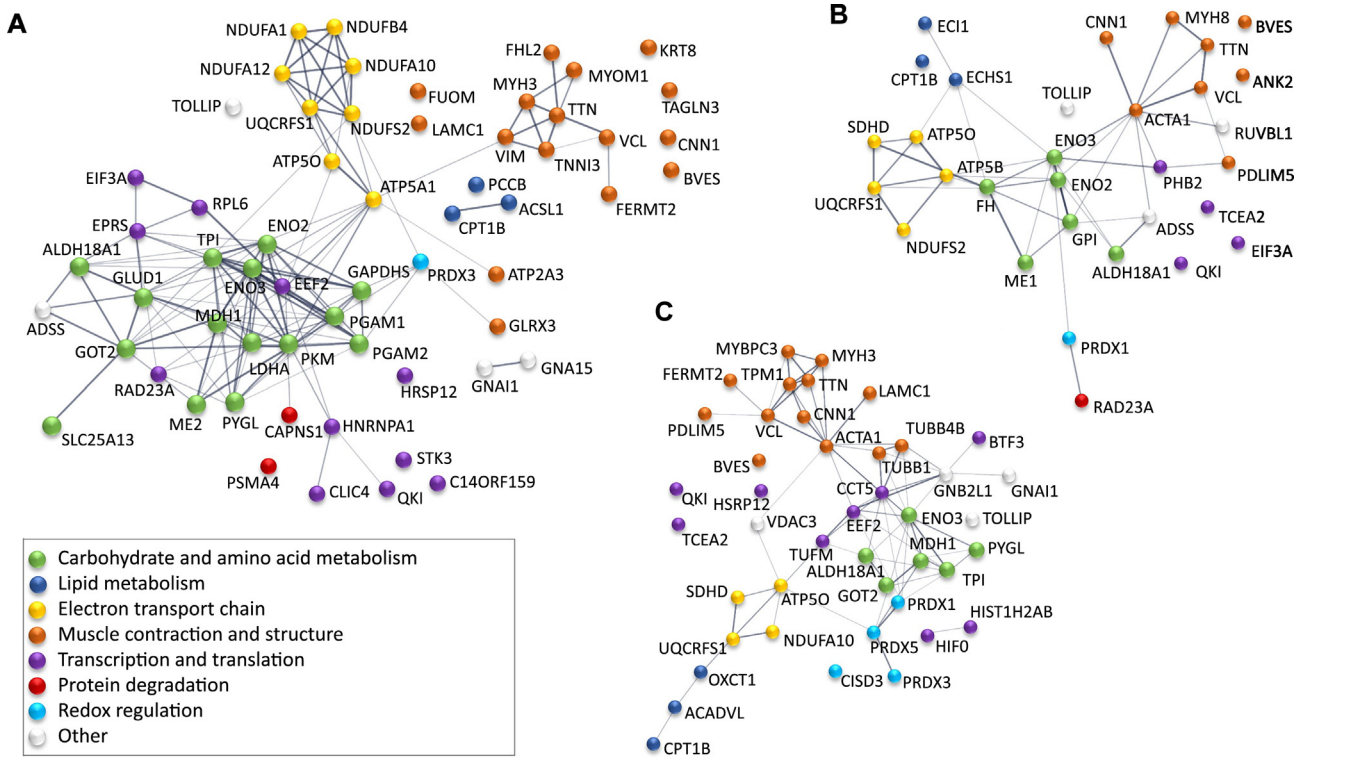

Fig. 4. Network analysis of protein-protein interactions among cardiac proteins that were significantly up or down regulated in the ventricles of fish during the early phases of exercise training at Day 4 (A), Day 7 (B), and Day 14 (C), relative to the ventricles of non-exercised fish at Day 0. Proteins are divided into 8 functional categories by colour and identified by gene name.

actin thin filament and myosin thick filament. This reaction is activated by Ca^{2+} binding to the troponin complex composed of TnI, TnC, and TnT, and leads to the shortening of the sarcomere. Like all proteins, those in the sarcomere are constantly turned over to replace those damaged by use. It can therefore be assumed that if the level of a protein in a cell remains constant over time, then the rate of replacement equals the rate of breakdown, and a change in a protein's abundance reflects a shift in this balance. Relative to fish at rest, forced swimming will accelerate contractile protein damage due to increased use, and this is reflected by the lower levels of α-actin and myosin-3 at each of the sampling days compared to the controls. Interestingly, the effect of sustained exercise on other contractile proteins does not follow this pattern. Many sarcomeric proteins increased in abundance during the swimming protocol, including TnI, titin and myomesin at Day 4, myosin-8 at Day 7, tropomyosin alpha-1 at Day 14, and a sustained increase in the thin filament-associated protein calponin at all 3 time points. This supports an early and sustained effort by the heart to counterbalance protein loss and maintain cardiac function.

Cardiac growth in adult fish is supported by both hyperplasia and hypertrophy [13]. Data from the present study supports exercise-induced hyperplasia, though hypertrophy cannot be ruled out. The fact that RVM is maintained through a 50% increase in body mass over 14 d indicates that cardiac growth rate is also increased in exercising fish. Contractile proteins are among the most abundant class of proteins in the heart, and with few exceptions, the abundances of these proteins either increase or are maintained at pre-exercise levels. To maintain relative proportions of contractile proteins during a period of cardiac growth implies the addition of new myocytes (cellular hyperplasia) with a similar protein complement as existing cells.

4.3. Markers of tissue integrity

Changes to structural connections between cells or within myocytes can have a significant consequence on tissue integrity as well as on how force generated by myocytes is translated to the contraction of the whole organ. After 4 d of exercise training, a clear increase in multiple structural proteins was observed, including proteins involved in building the cytoskeleton, and in linking the cytoskeleton to the sarcomere and the cell membrane. These changes are paralleled by increases in several myofilament proteins, as mentioned above, suggesting a concerted response by the heart to increase its force generating capacity. A reinforcement of intracellular connections was also observed, as indicated by the increase in vinculin isoform X2 and laminin subunit γ-1. These intracellular adhesion proteins would not only reinforce cardiac structural integrity but also enhance transmission of mechanical power, ultimately contributing to higher cardiac output. This early adaptive response is generally not maintained, despite the continuation of exercise training. By Day 7 and 14, many of these structural proteins return to Day 0 levels, and again this is paralleled by the changes in contractile proteins. There are two possible interpretations here. The first is that the cardiomyocytes have adapted to the increased workload and returned to the pre-exercise homeostatic balance of relative protein abundance needed to maintain cardiac function. The second is that cardiac integrity is weakened, as the increases in contractile and structural proteins needed to sustain the new level of cardiac function are not maintained after 4 d of exercise. However, given that vinculin-like isoform X2 and BVES are both significantly elevated at all time points relative to Day 0, and that BVES plays a conserved and definitive role in cell-cell adhesion in vertebrate myocytes [39], we propose that the former interpretation is more likely.

4.4. Energy metabolism

The cardiac output of an actively swimming fish is higher than that of resting fish to support the higher oxygen requirements of the exercising animal. In fish, cardiac output is primarily regulated by stroke volume, meaning more blood is pumped per beat. Such an increase requires greater force to be generated per contraction and therefore

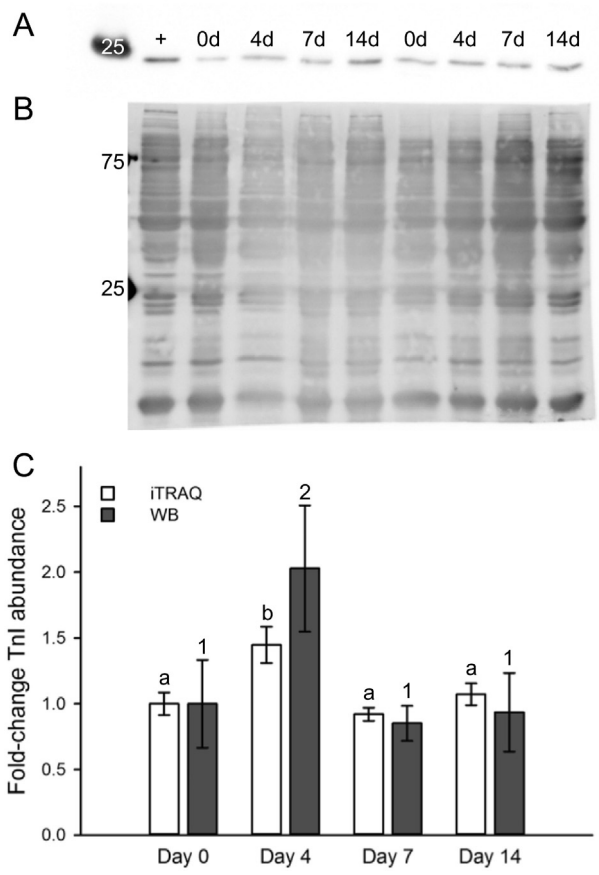

Fig. 5. Effect of exercise on cardiac troponin I (cTnI) abundance. (A) Representative Western blot image showing a single cTnI band at approximately 21 kDa for 2 biological replicates per time point, as well as an internal loading control (+) used to normalize across multiple blots. (B) Identical blot stained for total protein (SYPRO Ruby), and used to standardize densitometry quantification of cTnI. (C) Comparison of cTnI abundance quantified by iTRAQ (white bars, n = 3–4) or by Western blot (WB, grey bars, n = 2–4). Data is mean ± s.e.m. and bars that do not share a letter (iTRAQ) or number (WB) in common are significantly different.

the activation of more cross-bridges between actin and myosin. The end result is an increase in the ATP requirements for the heart. It is not surprising therefore, that training at moderate intensity generally leads to improved aerobic cardiac function supported by increased fatty acid oxidation, glycolytic activity, and oxidative phosphorylation. For example, rainbow trout trained at 40–60% U_{crit} for 4 wk exhibit an increased capacity for Kreb's Cycle, fatty acid oxidation, and glucose utilization, including increased activity of mitochondrial enzymes, and ultimately an increase in maximum cardiac power output [14,22]. Similarly, the hearts of Atlantic salmon exercised at 1.3 BL s^{-1} for 10 wk show an increase in the expression of genes involved in lipid metabolism as well as in mitochondrial density [12]. In sharp contrast to these studies, our analysis of the temporal changes to the trout cardiac proteome during the early phases of exercise training shows decreased abundance of enzymes involved in ATP production during at least the first 14 d of sustained, moderate-intensity swimming. This raises two important questions for further inquiry. First, is the observed decrease in the abundance of metabolic enzymes transient, declining during the acute phase of exercise training as protein stores are utilized and later increasing to yield the expected high aerobic-capacity phenotype described after 4 wk of training [14]? Second, is protein abundance a true reflection of pathway activity in the case of metabolic enzymes? Perhaps not, as a number of additional regulatory mechanisms, including phosphorylation and allosteric interactions of substrate:metabolite ratios could make up for any loss in pathway output arising from the decreased protein abundance. Either or both scenarios have merit for the present study, as it is unlikely that energy production within the heart is depressed considering that RVM was maintained as somatic growth accelerated during the 14 d exercise training program. Thus it does not appear that the decrease in proteins involved in aerobic metabolism is impacting the function or growth rate of the heart at this time point.

5. Conclusions

This comprehensive temporal analysis of the trout cardiac proteome during the acute phase of exercise training shows that the tissue remodelling response is initiated within days of increasing cardiac activity. This response includes an increase in contractile proteins and tissue integrity proteins as early as Day 4 to support increased force generation by the heart. Unexpectedly, the decreased protein abundance of metabolic enzymes did not reflect a gradual increase in ATP production capacity in the heart, which is a typical cardiac phenotype arising from extended exercise training. Since cardiac function was apparently not impaired by this protein decline, we suggest that regulation of metabolic pathways occurs at a higher order than protein expression, at least during the early training phase.

Transparency document

The Transparency document associated with this article can be found, in online version.

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