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ORIGINAL PAPER

Developmental rates, structural asymmetry, and metabolic fingerprints of steelhead trout (*Oncorhynchus mykiss*) eggs incubated at two temperatures

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Abstract Temperature stress on developing steelhead (*Oncorhynchus mykiss*) was evaluated using asymmetry of skeletal characters, fish condition factor, and metabolic fingerprints. Eggs from three female hatchery steelhead were fertilized by a single male. The eggs from each female were divided into two groups and incubated at either 8°C or 18°C. Mortality, growth, and condition factor were measured at stage 6 (32 cells), stage 20 (eyed), and stage 21 (caudal flexing). In addition, ^{1}H-nuclear magnetic resonance (NMR) spectroscopy was used to establish metabolic fingerprints of developing eggs at the three stages. After hatching, all alevins were moved to tanks at 18°C and allowed to develop to 60 days post-emergence (DPE), at which point they were examined for structural asymmetry. Eggs incubated at 18°C experienced higher mortality, with all eggs from one hen dying at the higher temperature. Eggs incubated at the higher temperature that did survive hatched as larger larval fish than eggs incubated at the lower temperature. Fish incubated at the higher temperature exhibited greater structural asymmetry than fish incubated at the lower temperature. A principle components (PC) analysis of the metabolic fingerprints indicated that PC1 and PC2 accounted for 60% of the variance in the metabolites. Separation along PC1 corresponded to differences in developmental stage, and separation along PC2 corresponded to differences in hen. Eggs incubated at 18°C lagged behind eggs incubated at 8°C along PC1, indicating a potential problem with embryo staging. PC1 scores were highly correlated with the accumulated thermal units during development, indicating that scores along PC1 were a robust measure of developmental stage.

Keywords Condition factor · Embryo · Metabolomics · NMR · Stress · Survival · Temperature

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Introduction

During reproduction, salmonids construct nests (redds) made of large gravel and cobbles in streams. Normal development of salmonid eggs and larvae is dependent on cold, well-oxygenated water flowing through the gravel (see review by Sullivan et al. 2000). The temperature range for optimal development varies across species but generally ranges between 8°C and 12°C (Sullivan et al. 2000). Temperatures above that range can cause a variety of structural and biochemical anomalies in developing embryos. Laboratory studies have demonstrated that salmonids incubated at higher temperatures generally have lower hatching weights (Baxter 1988), with higher incubation temperatures increasing egg and larvae metabolic costs and decreasing yolk utilization efficiency (Ojanguren et al. 1999). At higher temperatures the amount of unconsumed yolk in hatching Chinook salmon embryos was greater than at lower temperatures (Rombough 1994), raising questions of energy allocation and metabolic efficiency at higher growth rates during early stages of ontogeny. In Atlantic salmon, elevated water temperature during rearing has been found to increase anaerobic metabolism (Nathanailides 1996).

Outside of the normal temperature range for development, salmonid eggs experience both physiological and biochemical changes. Any of these biochemical or physiological changes may affect future development and survival (Bjorn and Reiser 1991). For example, Campbell et al. (1998) found that exposure to elevated temperatures during early development resulted in increased structural asymmetry in coho salmon (*Oncorhynchus kisutch*). Asymmetry within populations is often divided into three categories: fluctuating asymmetry, the small random fluctuations from perfect bilateral symmetry; directional asymmetry, the fixed asymmetry of one side; and antisymmetry, the random variation among members of a population resulting in a bimodal distribution of asymmetry (Palmer and Strobeck 1992). Fluctuating asymmetry has been shown to be a robust predictor of growth, survivability, and fecundity (see review in Møller and Shykoff 1999) and has been negatively correlated with fitness in rainbow trout (*Oncorhynchus mykiss*) (Leary et al. 1984). Aquatic ecologists have since used asymmetry to examine the health and stability of fish populations, including studies on the potential effects of inbreeding in salmonid broodstocks (Wagner 1996), recruitment in anchovies (*Engraulis encrasicolus*) (Somarakis et al 1997) and dietary differences in stickleback (*Gasterosteus aculeatus*) (Reimchen and Nosil 2001).

The effects of exposure to elevated temperature are reflected in an organism's metabolism and, consequently, on the metabolome, the full suite of endogenous metabolites that exist in an organism (Weckwerth 2003). During early development, the metabolome is expected to change as some metabolic pathways become activated and others are deactivated. If an organism experiences environmental stress, such as exposure to elevated temperature, metabolic pathways could be activated or deactivated prematurely or modified relative to the normal development. Analytical techniques such as ^{1}H-nuclear magnetic resonance (NMR) spectroscopy are now available to determine whether these changes are taking place and if they may have correlates with other developmental anomalies.

Although temperature speeds up both metabolic and growth rates, the cumulative affect of increased embryonic temperature on the biochemistry, physiology and structural morphology of fish larvae had not been examined within the same experiment. We exposed steelhead trout (*Oncorhynchus mykiss*) eggs to two different temperature regimes to examine the affect of rearing temperature on egg mortality, developmental rates, larval condition, egg metabolic fingerprints and structural asymmetry. Our objective was to investigate the combined biochemical and morphological response to temperature stress during development.

Methods

Animal incubations

Eggs were obtained from the Warm Springs Hatchery in Geyserville, California, USA from three different hens (H1, H2, H3) and fertilized

with the milt of one male. Approximately 3,000 fertilized eggs were transferred from the hatchery to the Center for Aquatic Biology and Aquaculture (CABA) at the University of California, Davis, immediately after fertilization. In addition, 50 unfertilized eggs from each hen were weighed and measured. The fertilized eggs from each hen were divided such that half were placed in incubation trays with water at 18°C (±0.2°C) and the other half in incubation trays with water chilled to 8°C (±0.75°C). CABA water is obtained from a local, ca. 60 m deep well and passed through a packed column aerator for re-oxygenation and to remove excess nitrogen. Water was passed through the incubation containers at flow rates of 5–7 l/min.

We were interested in examining the metabolic fingerprints of developing eggs at the same stage of development, even though those stages would occur at different times under different incubation temperatures. Consequently, we used IncubWin (http://www.pac.dfo-mpo.gc.ca/sci/aqua/sirp/incubwin_e.htm) to estimate when the eggs at the two incubation temperatures would reach a series of predetermined developmental stages. We checked the eggs several times daily to determine when the eggs had reached stages 6 (32 cells), 20 (eyed), 21 (caudal flexing), and when 50% had hatched. Timing of events in fish development is based on accumulated temperature units (ATUs), which are the accumulated number of degrees experienced by the eggs from fertilization. Given that the temperature–metabolic rate relationship is non-linear, we calculated ATUs for each sampling stage to determine if the stage–ATU relationship was similar between the two incubation temperatures.

Once the chorions of all the embryos had dissolved, and the alevins were able to move around, they were transferred into 400 l tanks with a water turnover rate of twice per hour with ambient temperatures of 18°C (±2°C). Embryos incubated at 8°C were acclimated to 18°C by our increasing the temperature every 12 h by 1°C over a 5-day period just prior to tank transfer. Once the yolk sacs had been absorbed, hereafter referred to as date of emergence, alevins were fed a semi-moist formulation (Rangen, Buhl, Idaho, USA: protein 50%, fat 16%, fiber 5%, ash 15%), to excess, twice a day. After a further 2–3 weeks of rearing at 18°C, fish were switched to a Silver Cup dry formulation (Nelson & Sons, Murray, Utah, USA: protein 45%, fat 11%, fiber 3%, ash 12%) and were fed twice a day at 3–7% total body weight.

Mortality, growth, and condition factor

Eggs were checked twice daily for any mortality; dead eggs were removed. Daily mortality was recorded for each temperature treatment until 30 days post-emergence (DPE). Weights and lengths were recorded for ten embryos in each group at emergence, 30 DPE and 60 DPE. Condition factor (K: weight/length^3 × 100,000) was calculated for each fish. No fish survived to emergence from H2 in the 18°C group, and, therefore, only fish from H1 and H3 have post-emergence growth data. At 174 DPE for the eggs incubated at 18°C, and 200 DPE for the eggs incubated at 8°C (229 days post-fertilization), ten fish from each tank were collected and given a lethal dose of buffered tricaine methanesulfonate (MS-222, 0.05%). Weights and lengths of the fish were recorded, and condition factor was calculated.

Structural asymmetry

We evaluated structural asymmetry at 60 DPE. Two individuals counted the numbers of pelvic rays (Pv), pectoral rays (Pt), brachiostegal rays (Br) and mandibular pores (Mand) on each side of the fishes' bodies. If counts for any fish differed, the count was repeated until there was mutual agreement on the number. Counts were made on 30 fish from each hen at each temperature. Fish from within each treatment were selected randomly for measurement. These fish were killed, cleared and stained, in accordance with the protocol outlined in Snyder and Muth (1990). The counts on the left side were subtracted from the right side (R–L) to determine the difference between sides for each trait. Although asymmetry should not have a genetic component, a two-way analysis of variance (ANOVA) was performed for each trait (response variable = difference in count between

left and right side), with hen and temperature as the main effects (Palmer and Strobeck 1992). To identify the presence of directional asymmetry, we performed one-sample *t*-tests on signed differences of each trait. Kurtosis values (g^2) below 0 provide evidence of anti-symmetry (Palmer and Strobeck 1992). An index of asymmetry was developed for each trait to summarize the amount of asymmetry at each temperature. The index is based on the Pearson product moment correlation (r) between the measurements of the right and left sides for each fish. If $1–r$ is used as the index for asymmetry, it is possible to determine the amount of variation in the trait that is not explained by asymmetry (Campbell et al. 1998; Leary et al. 1992; Windig and Nylin 2000).

Metabolic fingerprints

Twenty eggs from each hen and from each incubation temperature were removed and immediately frozen in liquid nitrogen at the following stages: stage 6, stage 20, and stage 21. Samples were stored at –80°C until required for extraction. Polar metabolites were extracted from whole eggs ($n = 4$ replicates from the 18 conditions, each consisting of five eggs) with perchloric acid, as described previously (Viant 2003), with the exception that 25 ml/g of perchloric acid was added to the dried tissue. Immediately prior to analysis the extracts were suspended in a 100 mM sodium phosphate buffer (in D_2O; pH 7.4) that contained 1 mM sodium 3-(trimethylsilyl)proprionate-2,2,3,3-d_4 (TMSP; Cambridge Isotope Laboratories, Andover, Massachusetts, USA) as an internal standard. ^{1}H-NMR spectra were measured at 500.11 MHz with an Avance DRX-500 spectrometer (Bruker, Fremont, California, USA) at 295 K. Specifically, one-dimensional (1-D) ^{1}H-NMR spectra of egg extracts were obtained using a 5.6 μs (60°) pulse, 7 kHz spectral width, and 2.5 s relaxation delay (with pre-saturation of the residual water resonance), with 120 transients collected into 32,000 data points, requiring a 10 min total acquisition time. All 1-D datasets were zero filled to 64,000 points, and exponential line broadenings of 0.5 Hz was applied before Fourier transformation. The resulting spectra were phase and baseline corrected and then calibrated (TMSP peak at 0.0 p.p.m.), all using XWINNMR software (version 3.1, Bruker). Peaks were assigned by comparison to tabulated chemical shifts (Fan 1996; Bollard et al. 2000; Sze and Jardetzky 1990).

The ^{1}H-NMR spectra were converted to an appropriate format for principal components analysis (PCA) using custom-written *ProMetab* software (Viant 2003) written in MATLAB (version 6.5; The MathWorks, Natick, Massachusetts, USA). Following the removal of the acetate, an unassigned metabolite, water and formate peaks (1.90–1.94 p.p.m., 3.40–3.45 p.p.m., 4.68–5.20 p.p.m. and 8.43–8.50 p.p.m., respectively) from each spectrum, because of extensive intensity or chemical shift variability, the remaining total spectral areas were normalized to one. The spectra were then subject to the generalized logarithmic transformation (transformation parameter, $\lambda = 1 \times 10^{-8}$; Purohit et al. 2004), which stabilizes the variance across the spectral data points and increases the weightings of the less intense peaks. Spectra were then divided into approximately 25 frequency regions, and the peaks within each region were aligned using a beam search algorithm (shift variable of 0.01 p.p.m., shrinkage variable of 0.00 p.p.m.; Lee and Woodruff 2004). Following concatenation of the 25 regions, the normalized, transformed and aligned spectra were segmented into 0.005 p.p.m. chemical shift bins. Bins between 7.05–7.10 p.p.m. and 7.78–7.88 p.p.m. containing pH-sensitive resonances were each compressed into single bins, resulting in a data matrix of dimension $65 \times 1{,}820$. Finally, the columns were mean-centered, and PCA was conducted with PLS_Toolbox (version 3.0; Eigenvector Research, Manson, WA, USA) within MATLAB. The resulting PCA scores plots served to visualize the metabolic similarities and differences between the egg extracts, and the loadings plots were used to identify metabolites that were dependent upon egg incubation temperature and upon maternal parent. One-way ANOVAs and Tukey–Kramer post-hoc tests were performed on the principal components (PC) scores derived from these analyses to evaluate the effects of hen and temperature on egg metabolic fingerprints, using Number Cruncher Statistical System (2001

Edition; NCSS Statistical Software, Kaysville, UT, USA). Finally, the relative concentrations of eight selected metabolites were obtained by integration of the binned data (without logarithmic transformation), and the same statistical tests were performed as above to test the effects of temperature, hen and developmental stage on the metabolite levels.

Results

Mortality

The initial number of eggs was not the same for all treatments and all hens. Consequently, survival was examined in two ways: mortality by date past fertilization (Fig. 1), and the cumulative mortality of eggs during the period of temperature exposure (Fig. 2). There were large differences among females in the ability of their eggs to withstand temperature stress. No eggs from H2 at the 18°C exposure survived to hatch. There appeared to be two periods of high egg mortality: the period between 4 days and 6 days post-fertilization at 18°C (approximately 65 ATU and 110 ATU) and a second period between 13 days and 18 days post-fertilization at 18°C (225 ATU and 325 ATU). Most of the eggs from H2 at 18°C died during the former period, and most of the mortality of eggs from the other two hens occurred at the latter stage, regardless of temperature.

To determine if there were significant differences in mortality among hens and between treatments, we performed a proportional hazards regression analysis, using the 1,104 eggs that died as observations of the response variable and temperature and hen as independent variables. Both independent variables were significant predictors of mortality (temperature—Z-value = 20.12, $P = 0.0000$; Hen—Z-value = –4.95, $P = 0.0000$) with a model pseudo-$r^2 = 0.44$.

Egg size and condition factor

Lengths of the hens varied slightly (fork length: H1 = 66 cm, H2 = 74 cm, H3 = 64 cm) but were not correlated with the observed differences in egg diameter, weight, or condition factor (Table 1). H3 was the smallest hen and produced eggs with the smallest mean diameter and weight but the highest condition factor. Condition factor was the smallest for the eggs from H2.

Egg development

Egg development relative to the ATUs was different for eggs reared at the two temperatures (Table 2). Eggs reared at 18°C were slightly

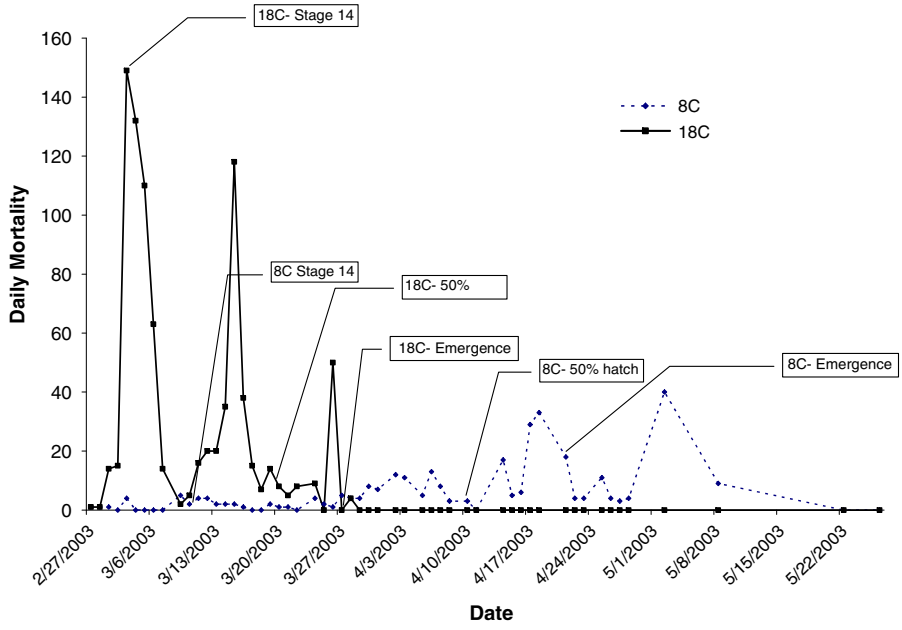

Fig. 1 Daily mortality of eggs during development

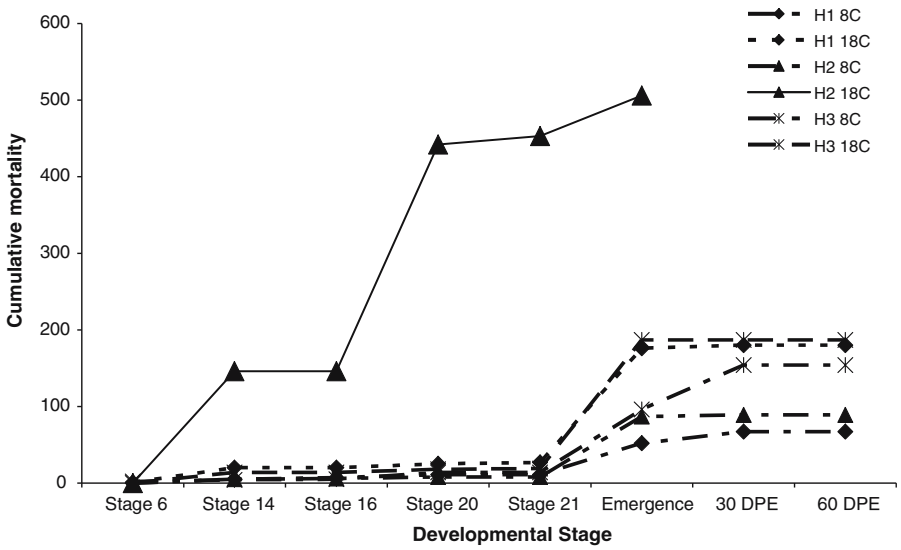

Fig. 2 Cumulative mortality of eggs during development

Table 1 Initial size of unfertilized eggs from the females used in the developmental study. All statistical tests to determine differences in egg parameters were Tukey–Kramer multiple comparison tests. Diameter was measured in millimeters, mass in milligrams, and condition factor (*K*) is unitless

Hen	Character	Mean	SD	Different from groups
H1	Diameter	5.04	0.52	H3
	Mass	101.3	3.88	H2, H3
	K	84.7	30.4	
H2	Diameter	5.02	0.29	H3
	Mass	97.7	4.74	H1, H3
	K	78.7	13.00	H3
H3	Diameter	4.35	0.35	H1, H2
	Mass	75.5	4.52	H1, H2
	K	95.2	22.34	H2

slower developing to stage 6 but accelerated thereafter. Eggs reared at 18°C reached stage 20 at 122 ATU, stage 21 at 148 ATU, and 50% hatch at 358 ATU. For eggs reared at 8°C, the ATU at the same stages were 181, 190, and 397.

Alevin development

Condition factors for larvae reared at two different temperatures were significantly different. A two-way ANOVA identified both hen ($F = 43.4$, df = 1,119, $P = 0.000$) and temperature ($F = 12.3$, df = 1,119, $P = 0.001$) as significant factors affecting condition factor for eggs from H1 and H3.

Table 2 The ATUs by stage for eggs incubated at 8°C and 18°C. The date is the date at which the eggs reached the stage listed. The last three columns are the ATUs at which the hens reached the stage

Temperature (°C)	Stage	Date	H1 ATU	H2 ATU	H3 ATU
18	6	2/27/03	10.20	10.20	10.16
18	20	3/06/03	122.88	122.81	122.40
18	21	3/10/03	148.37	148.35	147.86
18	50% hatch	3/20/03	357.61	358.78	357.89
8	6	2/28/03	8.21	8.29	8.19
8	20	3/21/03	181.41	183.09	180.36
8	21	3/22/03	189.47	191.29	188.42
8	50% hatch	4/14/03	397.15	401.12	395.38

The interaction term was not significant ($F = 0.1$, df = 1,119, $P = 0.71$). H2 could not be added into the analysis since only eggs from H2 reared at 8°C hatched.

Asymmetry

The two-way ANOVA for asymmetry in each trait indicated that there was no effect of hen on asymmetry, suggesting that no genetic component was present. All traits exhibited some level of

asymmetry at both temperatures, but the response to temperature was not consistent and suggests that increased temperature may cause chaotic perturbations during development. Steelhead eggs reared at 8°C exhibited fluctuating asymmetry (FA) in three out of the four traits measured, but the type of asymmetry changed to either directional or anti-symmetry with increased temperature. Only one trait, pelvic length, exhibited true fluctuating asymmetry in both temperature treatments (Table 3). Brachiostegal counts for fish reared at both 8°C and 18°C exhibited directional asymmetry. Pectoral length and the number of mandibular pores exhibited fluctuating asymmetry at 8°C but exhibited anti-symmetry at 18°C. With the exception of pectoral length, the index of asymmetry indicated that there was a higher level of asymmetry in all traits at 18°C (Table 4).

To determine if there were any relationships between asymmetry in any trait and fish performance, we calculated correlations between asymmetry and fish weight, length, and condition factor. No significant correlations were found.

Metabolic fingerprints

The first four principal components accounted for 73% of the variation in the NMR spectra, with PC1 and PC2 accounting for 37% and 23%, respectively. Both PC1 and PC2 had eigenvalues exceeding 1.0, indicating significance. The PCA scores plot of PC1 versus PC2 for all hens at all three stages demonstrated significant differences in the hens, stages, and temperatures (Fig. 3). Specifically, the three hens were well separated along PC2 at all developmental stages and temperatures, with increasingly positive PC2 scores associated with eggs with the lowest condition factor and highest mortality. The three developmental stages were clearly separated along PC1, with a trend for the scores to increase dramatically as eggs developed from stage 6 to stage 20, and then a smaller increase in PC1 between stages 20 and 21. PC2 scores remained relatively constant throughout all developmental stages. For each hen, there was no separation between temperatures for the first stage (stage 6). At both stages 20 and 21, however, there was a general trend for eggs incubated at 18°C to have (significantly) lower PC1 scores than eggs incubated at 8°C. Despite the fact that no eggs survived from H2 at the 18°C incubation, there was no indication of any grossly different pattern in the metabolic fingerprint relative to the other two hens through stage 21.

Using the scores on PC1 and on PC2 as the response variable, we performed two three-way ANOVAs with hens, temperature, and stage as the treatments. For PC1 scores there were significant differences between hens ($F = 266.5$, df = 2,65, $P = 0.000$), temperatures ($F = 269.5$, df = 1,65, $P = 0.000$), and stages ($F = 1382.8$, df = 2,65, $P = 0.000$). The hen by temperature interaction ($F = 0.8$, df = 2,65, $P = 0.44$) was not significant. The stage by hen ($F = 12.7$, df = 4,65, $P = 0.000$) and stage by temperature interactions ($F = 92.0$, df = 2,65, $P = 0.000$) were significant, and the three-way interaction was not significant ($F = 1.1$, df = 4,65, $P = 0.35$). For PC2 scores there were also significant differences between hens ($F = 531.6$, df = 2,65, $P = 0.000$), temperatures ($F = 12.7$, df = 1,65, $P = 0.001$), and stages ($F = 43.4$, df = 2,65, $P = 0.000$). The hen by temperature ($F = 5.1$, df = 2,65, $P = 0.010$), stage

Table 3 One-sample *t*-tests for each trait were measured against a mean of 0 to determine the type of asymmetry present. A significant difference (*) lends evidence that directional asymmetry is present. Kurtosis (g^2) of the (R–L) distributions of each trait is an indication of anti-symmetry if the value is less than 0

Trait	Temperature	*t*	*P*	g^2
Pv	8	0.000	–	7.244
Pt	8	1.904	0.060	3.148
Br	8	6.090*	0.000	–2.9136
Mand	8	1.101	0.274	0.477
Pv	18	–0.497	0.621	1.433
Pt	18	–0.903	0.370	–1.012
Br	18	3.3365*	0.001	2.399
Mand	18	0.000	–	–0.105

Table 4 Asymmetry index (1–*r*) of fish reared at 8°C and 18°C. The greater the index value, the greater the amount of asymmetry in the group of fish sampled

Temperature (°C)	Pv	Pt	Br	Mand
8	0.424	0.342	0.345	0.234
18	0.557	0.306	0.687	0.619

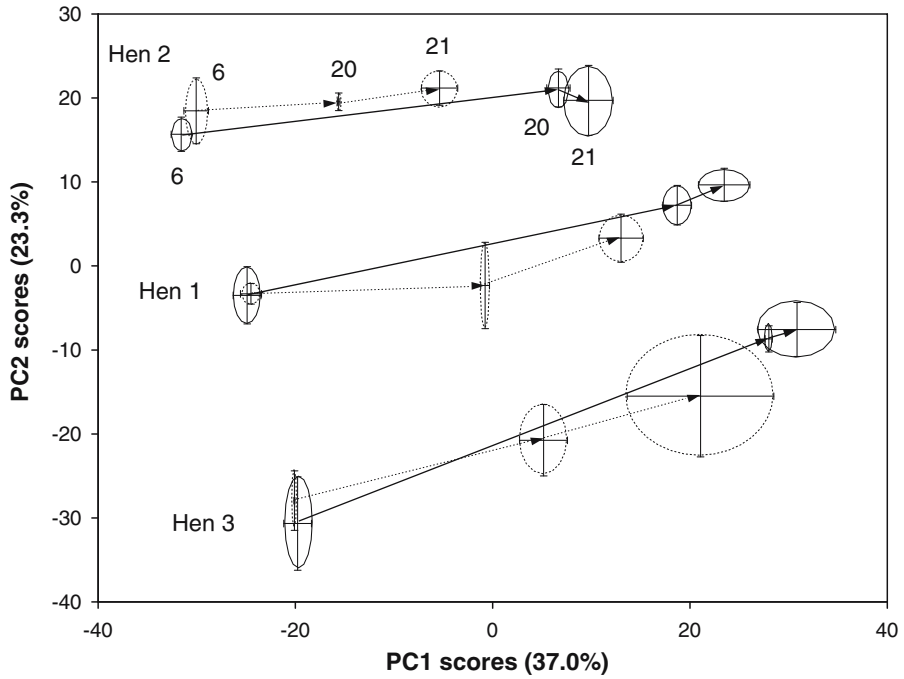

Fig. 3 PCA scores plot from a ^{1}H-NMR metabolomics analysis of steelhead egg development. *PC1* summarizes the metabolic changes occurring through development from stages 6 (32 cells; negative PC1 scores) to 20 (eyed) to 21 (caudal flexing), with each stage connected by an *arrow*. *PC2* summarizes the metabolic differences between the eggs from the three hens. An elevated incubation temperature (18°C, *dotted lines*) appears to retard normal metabolic development (8°C, *solid lines*). Each *ellipse* represents the mean ± SD of the PC1 and PC2 scores for each of the 18 groups of eggs (typically $n = 4$ per group)

by hen ($F = 7.5$, df = 4,65, $P = 0.000$) and stage by temperature interactions ($F = 8.9$, df = 2,65, $P = 0.001$) were all significant, but the three-way interaction was not significant ($F = 1.0$, df = 4,65, $P = 0.41$) (Table 5).

The PCA loadings plots for the PC1 axis, which mostly explains the metabolic changes during egg development, and for the PC2 axis, which mostly explains the metabolic differences between the eggs from different hens, are illustrated in Fig. 4a, b, respectively. Eight groups of peaks with relatively large loadings were selected, and their peak areas (corresponding to relative metabolite concentrations) were calculated. Of these, seven metabolites were positively identified as leucine (two doublets at 0.96 p.p.m. and 0.97 p.p.m.), valine (doublet at 1.05 p.p.m.), alanine (doublet at 1.48 p.p.m.), glutamate (multiplet at 2.35 p.p.m.), tyrosine (doublet at 6.91 p.p.m.), phenylalanine (multiplet at 7.42 p.p.m.) and glycine–betaine (correlated singlets at 3.27 p.p.m. and 3.91 p.p.m.). Our sample preparation technique does not lyse proteins, and, therefore, the amino acids observed are free metabolites. The remaining peak was tentatively identified as a choline-containing metabolite, most likely phosphocholine (singlet at 3.21 p.p.m.).

We performed eight three-way ANOVAs on these metabolites and a three-way MANOVA, using all eight metabolites to generate a response variable (Table 5). In the MANOVA and all the univariate ANOVAs, all main effects were highly significant. In addition, all interaction terms in the MANOVA were significant, and at least two interaction terms were significant in all univariate analyses, except for glutamate. The hen by temperature interaction term was significant in four of the univariate analyses, indicating that spectra changed differently across temperatures for the different hens. The hen by stage interaction term was significant in six of the seven univariate analyses, indicating that the spectra of the metabolites for each hen changed differentially across the stages.

Table 5 Multivariate analysis of variance (MANOVA) and ANOVA results for evaluating the effects of hen, temperature, and developmental stage on metabolite spectra. Results are given as Wilks' lambda test values (MANOVA) or *F* values (ANOVA), with associated probabilities in parentheses. Interaction terms are *H* hen, *T* temperature, *S* stage

Response variable	Source of variation			
	Hen	Temperature	Stage	Significant Interaction terms
Multivariate (MANOVA)	0.0066 (0.0000)	0.067 (0.0000)	0.0073 (0.0000)	H × T; H × S;T × S; H × T × S
Leucine	238.95 (0.0000)	5.42 (0.0243)	67.32 (0.0000)	H × T; H × S;T × S
Alanine	626.18 (0.0000)	10.22 (0.0025)	21.53 (0.0000)	H × T; H × S;T × S
Valine	395.06 (0.0000)	5.90 (0.0190)	40.08 (0.0000)	H × T; H × S
Glutamate	445.84 (0.0000)	11.26 (0.0016)	6.99 (0.0022)	-
Phosphocholine	4.97 (0.0111)	358.32 (0.0000)	640.02 (0.0000)	H × T; T × S;H × T × S
Glycine-betaine	4.08 (0.0232)	203.59 (0.0000)	627.92 (0.0000)	H × S; T × S
Tyrosine	16.04 (0.0000)	58.50 (0.0000)	296.52 (0.0000)	H × S; T × S
Phenylalanine	6.50 (0.0032)	108.16 (0.0000)	291.80 (0.0000)	H × S; T × S

Discussion

Survival and asymmetry

Like Campbell et al. (1998), we clearly demonstrated that thermal stress is linked to several effects, including increases in egg mortality, growth, and asymmetry of individual fish. In addition, we also demonstrated that thermal stress during development was linked to changes in metabolic processes, as determined by ^{1}H-NMR analysis.

Our design was unable to estimate a male contribution to the genetic variation in development, because we used a single male to fertilize all females in the experiment. However, there were large maternal effects in the survival of eggs and larval steelhead, which could have a genetic or maternal physiological basis. Although eggs from all three hens experienced reduced survival at 18°C, every egg from H2 died at this temperature. It is unlikely that the death of the eggs was a result of a factor(s) other than temperature, e.g., fungal infection. Non-viable eggs were removed from the incubation trays daily, and no fungal infection was noticed during the experiment. Also, eggs from all three hens raised at 18°C were in the same incubation chamber, and the mortality in the other trays of the chamber was much lower. Huuskonen et al. (2003) performed genetic crosses to determine the genetic contribution to temperature tolerance during development. They found that parental background affected only survival and not standard metabolic rate.

The relative range of tolerance of the eggs to elevated temperature is somewhat surprising, given the assumed homogeneity of the parental stock. All three hens were from the same hatchery stock and were harvested on the same day. Interestingly, the periods of time in which significant egg mortality took place did not vary among females. Although there was a small amount of mortality during all phases of development, the majority of the mortality took place at two periods. These periods loosely correspond to stages 19–21 and stages 23–24 and indicate periods when the eggs may be particularly susceptible to environmental stressors. The early stage corresponds to the period when the yolk sac is vascularized, while the latter is approximately centered on hatching.

For those eggs incubated at 18°C that survived, the rate of development was increased substantially compared to those reared at 8°C, with 50% hatch occurring almost a full month earlier in the 18°C eggs (Table 2). Emerging juveniles from 18°C incubations were larger than fish incubated at 8°C, although, after hatching, all fish were placed in 18°C tanks for rearing. Although differences were slight, fish incubated at 18°C had a higher average condition factor at emergence and at 30 days and 60 days post-emergence (Fig. 5), suggesting that the advantage in size is

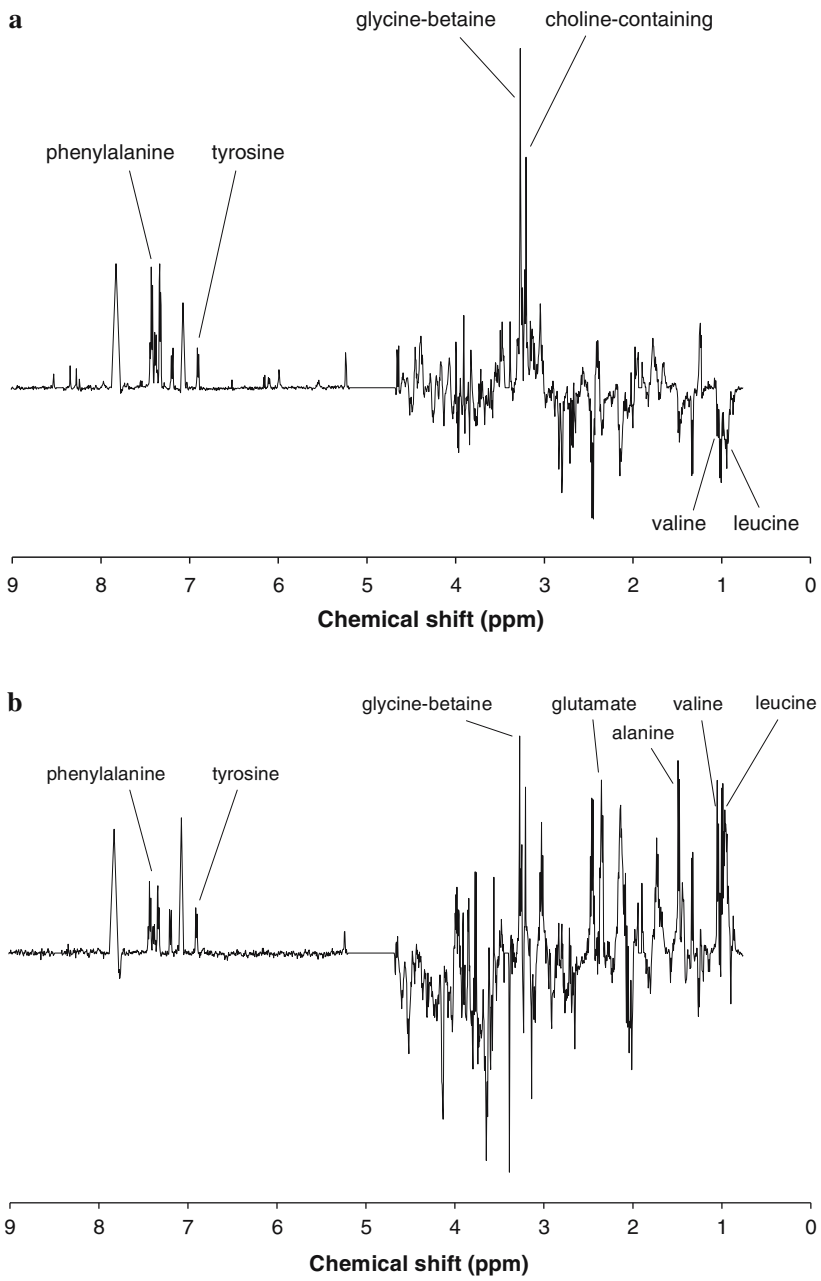

Fig. 4 PCA loadings plots from a ^{1}H-NMR metabolomics analysis of steelhead egg development, (**a**) for PC1 axis, where positive intensity peaks correspond to metabolites that are at elevated concentration in later stage embryos, and (**b**) for PC2 axis, where positive peaks correspond to metabolites that are at elevated concentrations in hen 3 and which are negatively correlated with condition factor

retained throughout larval development and into the juvenile stage. Interestingly, given the relatively fixed mass of the yolk sac, fish raised at 18°C were larger at emergence, prior to the initiation of exogenous feeding. The statistically significant differences in condition factor were a function of both differences in length and body mass, such that eggs incubated at 18°C resulted in longer and heavier alevins. During development, the only energetic costs are for maintenance of tissue that has already developed and growth of new tissue. Given a fixed amount of energy, if more energy is placed into growth, less is available for maintenance. The reduced energy for maintenance should result in changes in metabolic function as the developing embryo attempts to allocate optimally its energy resources.

Debate continues about the implications of using directional asymmetry and anti-symmetry as indicators of developmental instability. Van Valen (1962) hypothesized that directional asymmetry and anti-symmetry are genetically

controlled, because they do not show normal, bimodal distributions. However, fluctuating asymmetry may change to directional asymmetry if the amount of stress is sufficient (Palmer 1994). Leamy (1999) demonstrated that mandibular characters of mice that normally expressed fluctuating asymmetry changed to directional asymmetry when exposed to high doses of DDT. Of the ten characters expressing directional asymmetry in that study, only one had significant heritability. Our experimental design was too limited to determine if there was significant heritability in any of the characters, but, of the characters we selected for analysis, only brachiostegal counts have been found to have significant heritability (Jagoe and Haines 1985; Campbell et al. 1998; Bryden and Heath 2000). In our study, brachiostegal counts changed from fluctuating asymmetry to directional asymmetry after exposure to high temperatures during development, suggesting that there is an environmental component to the asymmetry.

Rate of development

Gillooly et al. (2002) recently developed a general model that predicts the time of ontogenetic development as a function of body mass and temperature. They generated equations for the change in mass of an organism as a function of the organisms' asymptotic mass, fundamental cellular properties and the metabolic rate. They developed a series of plots that established the relationship between mass-corrected development time and temperature. If we assume that the asymptotic mass of all eggs of the same species is the same, the only factor controlling development rate is temperature. The expression relating development time (D) to temperature (T_c) is

$$D = T_c/(1 + (T_c/273))$$

Substitution of the 8°C and 18°C temperatures into the expression predicts that the development times to any stage should be 2.17 times faster at 18°C than at 8°C. The ratio (18°C/8°C) of the time to stage 6 is 0.91, to stage 20 is 2.88, to stage 21 is 2.67, and to 50% hatch is 2.14. We observed an average increase in development time across embryonic development for the 18°C eggs relative to the 8°C eggs of 2.13, very close to the predicted ratio of 2.17.

Metabolic fingerprints

The PCA scores plot from the metabolomics analysis summarizes the changes in all the NMR-observable metabolites during development. For each of the hens, the metabolic fingerprints of the stage 6 eggs are the same at incubation temperatures of 8°C and 18°C. This

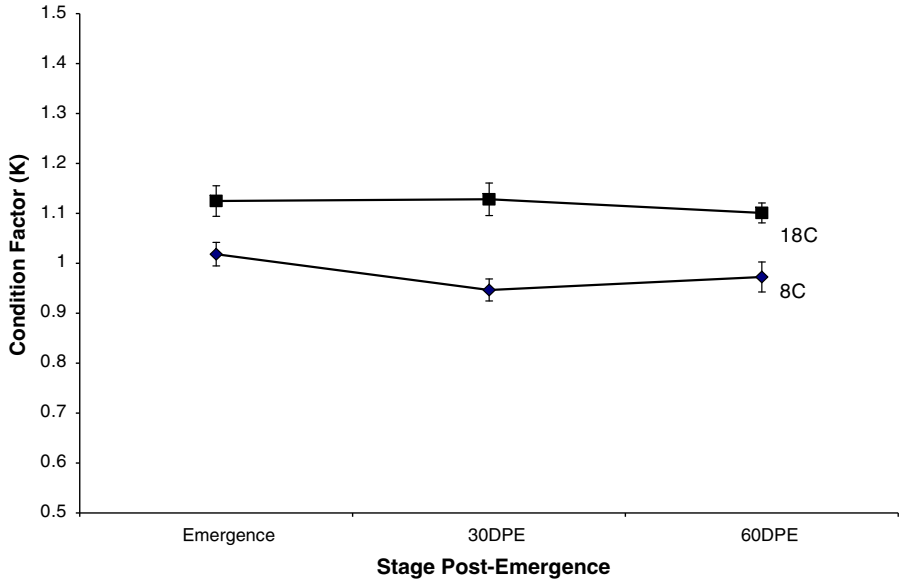

Fig. 5 Condition factor of fish at emergence, 30 DPE, and 60 DPE from eggs incubated at 8°C and 18°C. *Error bars* are standard errors. Data are combined across hens for each temperature, but, for eggs reared at 18°C, no eggs from H2 were included

is likely because, at this very early 32-cell stage of development, the metabolite fingerprints of the whole eggs are dominated by the composition of the yolk that is insensitive to the incubation temperature. Considerable metabolic changes occur between stages 6 and 20, as evidenced in the scores plot, and this change is significantly greater in the 8°C groups. This suggests that a stage 20 embryo developing at 8°C is metabolically more developed than a stage 20 embryo at 18°C. A similar effect is observed between stages 20 and 21, where a small metabolic change occurs at an incubation temperature of 8°C, as may be expected for two closely related developmental stages. At 18°C, although the change between stages 20 and 21 is larger than at 8°C, the stage 21 embryos developing at 18°C are still less metabolically developed than those at the lower incubation temperature. These results are apparently not consistent with the well-established increase in metabolic development expected at the elevated incubation temperature (Gillooly et al. 2002). We must, therefore, consider the accuracy of the staging methods employed in the current study, which were estimated using the IncubWin software. A logical interpretation of the PCA scores is that the eggs incubated at 18°C were estimated to develop more rapidly than actually occurred, i.e., when the eggs were harvested at the estimated stage 20 they were, in fact, at stage 19 or earlier. This would then explain why their metabolic fingerprints (in the scores plot) lie to a less developed stage (smaller PC1 scores) than the corresponding 8°C groups. However, the difference between stage 19 and stage 20 is in the development of eyes, a relatively easy structure to identify. Also, for this explanation to be correct, the accuracy of the staging of eggs incubated at 18°C would have to be lower than that of staging of eggs incubated at 8°C. Even though we used stages 20 and 21 as developmental markers for evaluating metabolic fingerprints, the two stages are actually separated by a considerable number of ATUs (Table 2) and several days of development. Significant development occurs between the two stages. It is also unlikely that fish at stage 20 could be mistaken for fish at stage 21.

Alternatively, the staging was accurate and the eggs incubated at elevated temperatures were less well developed metabolically than those incubated at lower temperatures. Although it is possible that the staging may not be exact, it is unlikely that incorrect staging accounts for all of the difference in metabolic fingerprints, and, consequently, the differences in PC scores can be attributed to the effects of elevated incubation temperature.

In Fig. 6 the average PC1 scores for each stage of development are highly correlated with the measured ATU for each hen. This is not only a

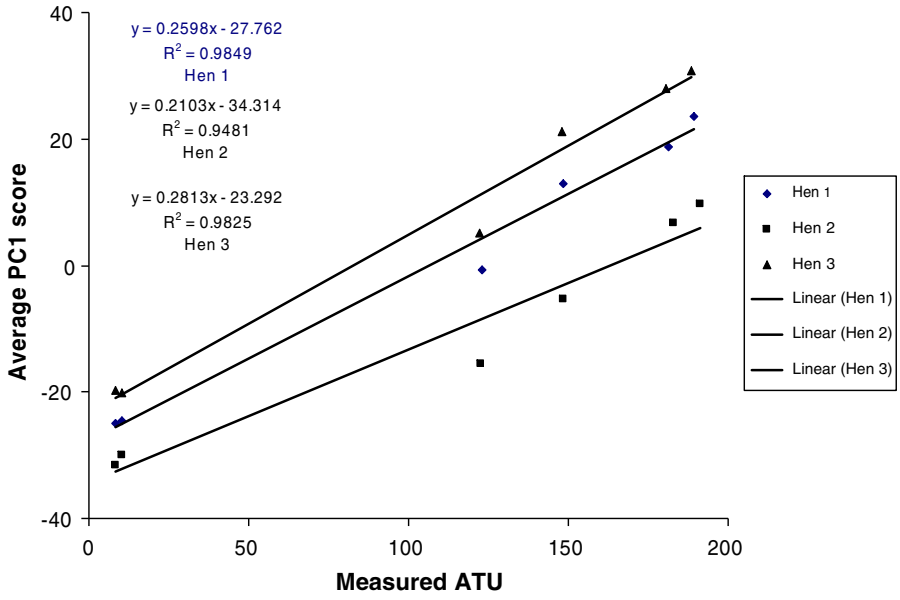

Fig. 6 Relationship between accumulated thermal units (ATUs) and average score on the PC1 axis for eggs incubated at 8°C and 18°C

reassuringly anticipated result, but it illustrates the power of the PC1 score, derived from the NMR metabolic fingerprints, in providing a robust measure of developmental stage. We have observed a similar result across nine developmental stages in the Japanese medaka (Viant 2003).

An analysis of the PCA scores plot (Fig. 3) and loadings plot for PC2 (Fig. 4b) can provide insight into the metabolic differences between healthy and potentially compromised eggs. Interestingly, the metabolic differences between the embryos from H1, H2 and H3 that are undergoing caudal flexing (stage 21) are very similar to the metabolic differences between the embryos from H1, H2 and H3 in the 32-cell stage. This emphasizes the importance of the physiological condition of newly fertilized eggs in determining the metabolic status of embryos just prior to hatch. Metabolic differences of particular significance are the elevated concentrations of alanine and glutamate in the eggs from H2 versus H1 and H3, which occur at all three developmental stages. Furthermore, since these amino acids show minimal temperature dependence, we can conclude that elevated levels of alanine and glutamate appear to be correlated with a low egg condition factor. The origin of this relationship is unknown. A somewhat similar effect is evident for leucine and valine, both of which are elevated in the eggs from H2. These amino acids, however, have a tendency to decrease with stage of development (for H1 and H2), and this effect is exaggerated for the eggs incubated at 18°C. This observation is in contrast to the increase in free leucine and valine concentrations of both cultured and wild Atlantic salmon eggs (*Salmo salar*) between fertilization and the eyed stage (Srivastava et al. 1995).

Concentrations of the aromatic amino acids tyrosine and phenylalanine exhibit an even more complex behavior, exhibiting obvious effects due to hen, stage of development and temperature. For example, H2 eggs with the lowest condition factor have the highest concentrations at stage 6 but show the smallest increases throughout development. Both metabolites also exhibit a reduced concentration increase in eggs incubated at 18°C. The overall increase in tyrosine and phenylalanine levels through development is consistent with studies of developing Atlantic salmon eggs (Srivastava et al. 1995).

Other metabolite concentrations appear independent of hen, such as that for glycine–betaine and phosphocholine (tentative assignment). These metabolites instead exhibit a significant increase during development at 8°C, increasing 2.7-fold and 2.4-fold between stages 6 and 21, respectively. An elevated incubation temperature, however, apparently reduces these increases to only 2.0-fold and 1.7-fold, for glycine–betaine and phosphocholine, respectively.

If asymmetry is the result of developmental instability, there should be a relationship between temperature, asymmetry, and specific metabolic pathways that are active during the time of bone formation and growth. A first step in establishing this connection is to identify differences in metabolic fingerprints between individuals that are exposed to different temperatures. Unfortunately, it is not possible to link asymmetry of individuals directly with their metabolic fingerprint during development, because of the difference in time of sampling for the two types of data. However, we did sample fish for asymmetry and metabolic profiling from the same exposure regime and found significant differences in metabolic status within the set of metabolites observed by ^{1}H-NMR.

Overall, our results demonstrate the utility of NMR metabolomics for rapidly assessing the metabolic changes occurring during steelhead embryo development and relating those changes to other adverse effects of temperature. The PCA scores plot provides a succinct and simple 2-D representation that summarizes changes in the entire NMR-observable metabolic profile, and the loads plots enable interpretation of precise metabolic changes. A more comprehensive interpretation of these changes awaits the on-going development of methods to assign the majority of peaks within the NMR spectra.

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