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Research Article

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Changes in Metabolic Rate, Excretion, Energy Reserves, and Starvation Response of Diploid and Triploid *Salvelinus Fontinalis* and Diploid *O. Mykiss* after Long-Term Exposure to Elevated Temperature

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Abstract

Background: *In fish culture metabolic rate (oxygen consumption), and excretion of particular and dissolved waste products are important parameters limiting the production capacity and influencing the environment. Data on their changes in response to elevated temperature are important for future management of fish farms. Therefore, these parameters were investigated in diploid (2n) and triploid (3n) Salvelinus fontinalis and 2n Oncorhynchus mykiss exposed to 20°C for 32 d in comparison to fish acclimated to 9 °C. Additionally energy reserves were investigated as important indicators for fish performance.*

Results: *At 20°C routine metabolic rate (RMR) of 2n S. fontinalis decreased with increasing exposure time and after 32 d it did not differ from 9 °C. Also, in O. mykiss RMR decreased with exposure time but remained 60 % higher than at 9°C. In 3n S. fontinalis exposed to 20°C RMR was constantly increased for 120%. For all species/ploidy levels faeces dry weight and phosphorus concentration and quantities of excreted dissolved nitrogen and phosphate did not differ between 9°C and 20°C. Lipid concentration of faeces was decreased at 20°C. With exception of O. mykiss also faeces protein was decreased at 20°C. In all species/ploidy levels liver glycogen and visceral fat was decreased at 20°C, while liver triglycerides and adenylate energy charge were not affected. In 2n and 3n S. Salvelinus temperature related responses to 5-d starvation were investigated. In 2n S. fontinalis RMR decreased in starving fish in comparison to fed ones at 9°C and increased at 20°C. RMR of 3n S. fontinalis did not change. For both ploidy levels, dissolved excretion was decreased due to starvation at 9°C and 20°C. Visceral fat decreased due to starving at 9°C, liver glycogen and triglycerides at 20°C.*

Conclusion: *In Salmonidae species and ploidy dependent types of metabolic thermal compensation occurred after exposure to 20°C. Effects on waste products were minor for all investigated species and ploidy levels. Exposure to 20°C represented an energetic stress situation as the visceral fat and liver glycogen depots were depleted. Consequently, at elevated temperature starving can result in a critical energetic stress situation which might lead to complete exhaustion of energy resources.*

Keywords: Routine metabolic rate, excretion, faeces, starvation, energy status, adenylate energy charge

Background

Abrupt exposure of teleost fish species to a sudden temperature change leads to thermal shock reactions, while gradual exposure in combination with sufficient long exposure time induces acclimatization reactions [1, 2].

Knowledge about acclimatization processes is important in ecology and fish culture to define the plasticity of teleost fish species to temperature changes [2]. The acclimatization potential of economically valuable salmonid species is of particular interest in Austria as well as in many other temperate regions.

In fish culture oxygen consumption (= metabolic rate) and excretion of particular and dissolved waste products are important parameters limiting the production capacity and influencing the environment [3]. Data on their changes in response to elevated temperature are important for future management of fish farms [4-10]. Standard metabolic rate of teleost fish increases, when they are abruptly exposed to elevated temperature (4-11). However, only little is known if and to which extent standard metabolic rate can be reregulated to acclimation conditions in form of a partial or total metabolic compensation. Standard metabolic rate of O. mykiss acclimated to 9°C and exposed to 20°C was partially re-regulated to acclimation conditions after 72 - 96 h [12]. Shorthorn sculpin (*Myoxocephalus scorpius*) showed total metabolic compensation following a temperature increase from 9°C to 16°C after eight weeks of acclimation [13]. Similar results were also found in Atlantic halibut (*Hippoglossus hippoglossus*) after long-term exposure to 16°C [14].

Only few data are available how the composition of particular excretion (faeces) and the quantity of dissolved excretion are affected by elevated temperature [15-17]. Digestion processes decrease at temperatures outside the optimal range due to changes in enzyme activities and in gut transit time; Atlantic salmon, *Salmo salar, Salmo trutta*, Arctic charr, *Salvelinus alpinus*, - [18]. Differences in food digestibility could lead to alterations in faeces composition. Excretion of dissolved ammonium depends on fish activity levels, diet, and protein catabolism [19, 20]. Quantities of excretion show a positive relation with temperature in several teleost fish species [21 - 24]. In fed *O. mykiss* [25, 26] no relation was detected between ammonium excretion and temperature in but it was positively correlated with temperature in walleye, *Stizostedion vitreum,* [21-27]. Phosphorus excretion depends on the administered chemical form, the diet, the growth rate, and the body size [28]. In a natural ecosystem, temperature had a minor influence on fish phosphorus excretion rates [29]. In Silver perch, *Bidyanus bidyanus*, the phosphorus excretion was significantly increased at elevated temperature [30, 31].

The present study investigated if the routine metabolic rate (RMR), the composition of faeces (dry mass, protein, lipid, and phosphate content), and the quantities of excreted ammonium, total nitrogen bound, and phosphate of salmonid fish kept at 20°C for 32 d differed from fish acclimated to 9 °C. To get an overall picture about fish physiology, energy reserves of (liver and muscle glycogen, liver triglycerides, muscle total lipids), visceral fat depots, and cell energy status (cellular ATP concentration, adenylate energy charge) were investigated, too. For the investigations diploid (2n) and triploid (3n) brook trout *Salvelinus fontinalis* and 2n *O. mykiss* were used as these species are of high economic value and as this study complements previous ones on adaptation processes to elevated temperature [32, 33]. In fish culture, it is a common practice to reduce or stop feeding at elevated temperature to decrease oxygen depletion and excretion load of water and therefore to maintain stable water conditions [34, 35, 36]. Therefore, in 2n and 3n *S. Salvelinus* temperature related effects of a 5-d starving period on routine metabolic rate, quantities of dissolved excretion, and energy reserves were investigated in the end of the temperature exposure experiment.

Methods

The used experimental fish were 7 months old 2n O. mykiss (initial body mass 8 ± 3 g, n = 30, mean ± S.D.), 2n *S. fontinalis* (9 ± 3 g), and *3n S. fontinalis* (10 ± 4 g). The latter were produced by pressure shock. Experimental fish and experimental design were similar to a previously published study [32]. Briefly, experiments were conducted from July 12–August 12 2021 in stream channels under flow through conditions with a water supply of 0.2 l sec-1. Four stream channels, respectively, were stocked with 2n rainbow trout, and 2n and 3n brook trout. Total stocked fish mass was 3 kg per stream channel resulting in approximately 200 fish. For each species/ploidy level two stream channel were maintained at 9 °C (= controls). The other stream channels were gradually tempered to 20 °C during a 7-d period. When the water temperature had reached 20 °C, the experiment was started. The duration of the experiment was 37 d. Fish had a natural photoperiod and were fed a commercial trout diet (protein 39 – 41 %, lipid 19 – 22 %, raw fiber 1.0 - 2.0 %, ash 4 - 8 %, phosphorus 0.87 %) at a ratio of 1.5 % of the body weight using band feeders during daylight time. Similar quantities of feed were administered at both tested temperature regimes. Loss of equilibrium was used as an endpoint of critical thermal stress experiments [33]. The rate of fish maintaining equilibrium was calculated at the end of the experiments in relation to the total number of fish stocked in the stream channels. Hygienic concepts and screening methods for water quality and fish health status were described previously [32]. Experiments were carried out in accordance with Austrian regulations governing animal welfare and protection and with the EU directive 2010/63/EU for animal experiments.

Analysis Design

The analysis design is illustrated in Figure 1. After exposure periods of 8, 16, and 32 d to 20°C RMR was measured in 2n O. mykiss and 2n and 3n S. fontinalis in comparison to control fish kept under similar conditions but at 9°C. Quantities of dissolved excretion, composition of particular excretion and fish energy reserves and energy status were measured on day 32 d. From day 33 to 37 feed-

ing was stopped. On day 37 routine metabolic rate, concentration of dissolved excretion and fish energy reserves and energy status and were remeasured.

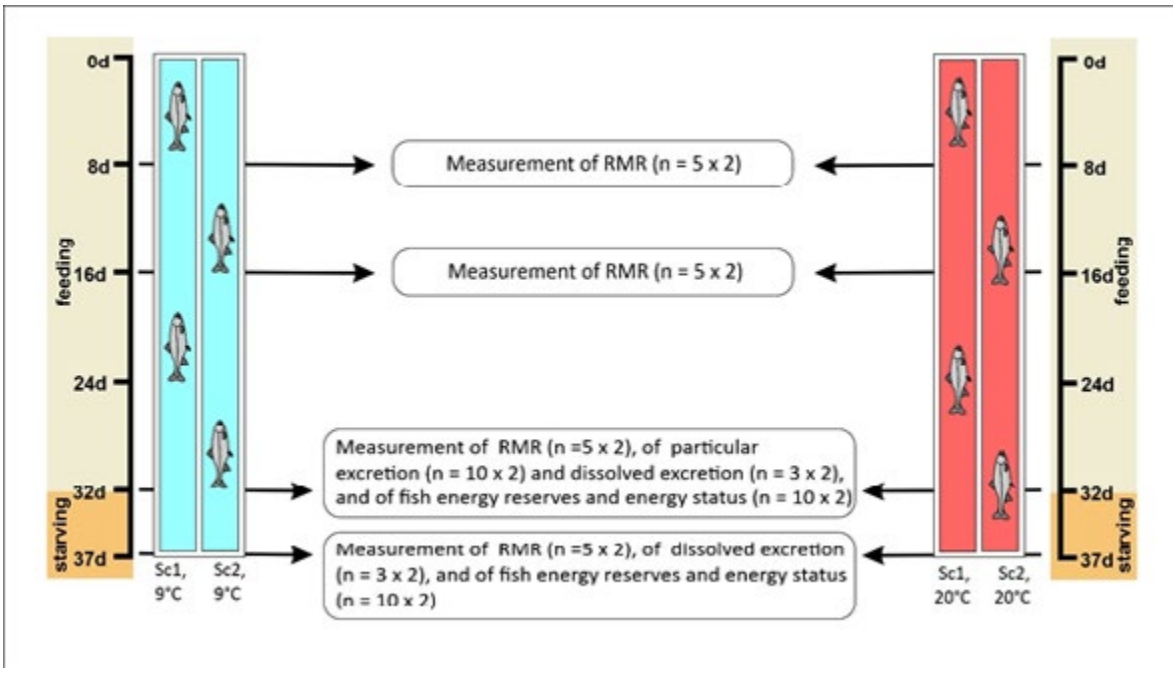

Figure 1: Scheme of the Experimental Design. SC – Stream Channel, RMR -Routine Metabolic Rate

Analysis of Particular and Dissolved Excretion

For analysis of particular excretion, 10 fish per stream channel were killed by prolonged exposure to 0.3 % MS222 six hours after they had started feeding (i. e. 6 h after the belt feeders had been switched on). The abdominal cavity was opened and an amount of 20-25 mg faeces was collected from the rectum. The faeces samples were split in 2 subsamples and their mass was determined to the nearest 0.1 mg using an analytic balance. One subsample was used for determination of dry weight and total phosphate concentration, the other subsample for protein and lipid determination. For determination of dry mass, the subsample was heated to 100°C for 24 h and reweighed to the nearest 0.1 mg. Then the dry matter was digested in 200 µl 1 mol l-1 HCl in 1 ml screwed vials at 105°C for 4 h. The samples were cooled down and phosphate was determined according to standard methods [37]. From the second subsample lipids were extracted with chloroform methanol [38]. In the chloroform methanol extract total lipid was determined with the sulphuric acid - vanillin method [39]. The lipid free sample was used for total protein determination. Total protein was extracted with 500 µl of 0.1 mol l-1 NaOH in 3.5 % NaCl at 60 °C for 90 min [40]. The homogenates were centrifugated at 5000 g for 10 min to remove insoluble particles. Protein was analyzed with the Lowry method [41].

For analysis of dissolved excretion 5 fish, respectively, were removed from the stream channels 6 h after they had started feeding and placed in a static 15 l tank of similar water temperature. Tanks were aerated with air stones using aquarium pumps. After 24 h the fish were removed from the tanks and their mass was determined to the nearest 0.1 g. 1-l water samples were taken according to standardized sampling procedures and frozen at -20°C until analysis. For each stream channel, the experiment was performed in triplicate. Standardized methods were used for determination of NH4-N, NO2-N, NO3-N and orthophosphate [42-45]. Total nitrogen bound was calculated as the sum of NH4-N, NO2-N, and NO3-N nitrogen. All analytes concentration were expressed in unit's kg fish-1 h-1.

Determination of Routine Metabolic Rate

Routine metabolic rate is the rate of metabolism when the fish is undergoing behaviors normal to fish farm conditions. A closed rectangular respirometer chamber with a volume of 4 l was used which could be operated under flow through conditions by connecting it via valves to the water supply of the fish farm and under recirculation conditions by connecting it to a recirculation pump. The whole recirculation system had a volume of 8.5 l. The flow volume was 0.4 l sec-1 resulting in velocities of flow of 0.01 m sec-1 in all operation modes. Flow through conditions were used during the acclimation phase and for water renewal after the measurements. The recirculation mode was used during the oxygen measurements. Oxygen concentration was measured using an optic oxygen sensor (WTW FDO 925) sealed within the chamber. It was connected to a central control unit logging the oxygen concentration. Five measurements per stream channel were performed.

The operation procedure was as follows: The respiratory chamber was switched to flow through conditions, and stocked with 3 fish. After 20 min acclimation under flow through conditions the respiration chamber was switched to recirculation conditions and oxygen measurements were started. Oxygen consumption was recorded for 30 min in 5 min intervals. Only measurements were used where the decrease in oxygen consumption was linear which was considered as an indication that fish did not falsify results due to short-term stress responses or activity changes. After the experiments were finished, fish mass was weighed to the nearest 0.01 g. Total oxygen consumption in the respirometer chamber per time unit was calculated and extrapolated to express the oxygen consumption in mg O2 h-1 kg fish-1. For measurement of standard metabolic rates reintermittent-flow respirometry persisting over 24 – 48 h is an alternative to provide stressless conditions [46, 47]. However, these procedures were not applicable in combination with the used experimental design. Long measurement periods would have interacted with feeding regimes and photoperiod and could have induced uncontrollable microbiological growth resulting in falsified oxygen values. Moreover, RMR may better reflect the conditions of fish farms, where fish cannot be maintained in stressless environment.

Analysis of Fish Energy Reserves and Cell Energy Status

Liver and viscera (digestive tract without liver) mass was determined. Liver subsamples were weighed in 1.5-mL microcentrifuge tubes with an analytical balance to obtain a reference unit for the metabolic measurements. Muscle tissue was collected from the right side of each fish, above the lateral line, and between the dorsal and caudal fins. Metabolites were extracted into 3 mol l-1 perchloric acid. Samples were homogenized and kept in extraction solution for 15 min under constant agitation, centrifuged at 1500 g for 10 min and the supernatants were collected. Finally, supernatants were neutralized using 1 mol l-1 potassium carbonate. Glycogen, triglycerides, ATP, AMP, and ATP were measured UV-spectrophotometrically [48]. Total lipid was detected as described above [39]. Adenylate energy charge (AEC) was calculated according to the formula

$$AEC = \frac{Conc_{ATP} + 0.5 * Conc_{ADP}}{Conc_{ATP} + Conc_{ADP} + Conc_{AMP}}$$

From the viscera lipids were extracted according to the procedure of Bligh and Dyer [38]. The solvent was evaporated at 70°C and the extracted fat was determined gravimetrically. Liver glycogen and triglycerides and visceral fat are energy resources for the fish. Therefore, to compensate for differences in organ and body mass, the total concentration in the organ was calculated and referred to 100 g body mass. Liver ATP levels and adenylate energy charge were used to characterize the energy status of cells and referred to a defined organ mass.

Statistics

Data are presented in form of boxplots. The solid lines represent the medians, boxes represent lower and upper quartiles, and whiskers the minima and maxima, circles the outliers. Percentage data (hepatosomatic index) were transformed by angular transformation (arcsin√p). For statistical analysis continuous data were tested on normal distribution by Shapiro-Wilk test and transformed by log transformation when necessary. Data were analyzed by two-way ANOVA with treatment and species as independent variables and analytes values as dependent variables. Tukey test was used as post-hoc test at a significance level of $P < 0.05$. Statistical analysis was performed with JASP software [49].

Result

Fish Equilibrium Rate and Growth

A percentage of > 95% of 2n and 3n *S. fontinalis* and 2n *O. mykiss* exposed to 20°C for 32d and a percentage of > 96 % of control fish acclimated to 9°C maintained equilibrium. Control fish ingested the total amount of administered food during the whole duration of the experiment. Fish exposed to 20°C leftover some part of the food during the first 8 d of the experiments, thereafter the total amount of administered food was ingested, too. In 2n, *S. fontinalis* kept at 9°C body mass increased for 164.0 ± 7.2 % during the duration of the experiment, at 20°C for 214.8 ± 8.8 %. In 3n *S. fontinalis* it increased for 181.3 ± 8.8 % at 9°C and for 203.3 ± 7.8 % at 20°C. In *O. mykiss*, the increase in body mass amounted 176.2 ± 6.5 % at 9°C and 139.5 ± 7.9 % at 20°C. 2n and 3 n *S. fontinalis* tolerated also a 5-d starving period at both investigated temperatures (percentage of fish maintaining equilibrium > 98%). In 2n *S. fontinalis* the loss in body mass was 3.5 ± 1.4 % at 9°C and 4.5 ± 2.1 % at 20°C after the 5-d starving period. In 3n *S. fontinalis* it was 4.3 ± 1.7 % and 3.5 ± 2.6 % at 9°C and at 20°C. The differences were not statistically significant ($P > 0.05$).

Routine Metabolic Rate (RMR)

Differences between 9°C and 20°C (Fig. 2)

After 8 d exposure to 20°C RMR of 2n *S. fontinalis* was significantly higher (for 50%, $P < 0.05$) than at 9°C. It decreased with increasing exposure time and after 32 d it did not differ any more from 9 °C ($P > 0.05$). At 9°C RMR of 3n *S. fontinalis* was significantly lower than that of 2n *S. fontinalis*. In 3n *S. fontinalis* exposed to 20°C RMR was significantly higher (for 120%) than at 9°C and it did not change during the exposure period. Also, in O. mykiss RMR was significantly higher ($P < 0.05$) at 20°C than at 9°C (for 110% after 8d). It decreased with exposure time but remained higher (for 60%) than in 9°C fish after 32 d ($P > 0.05$).

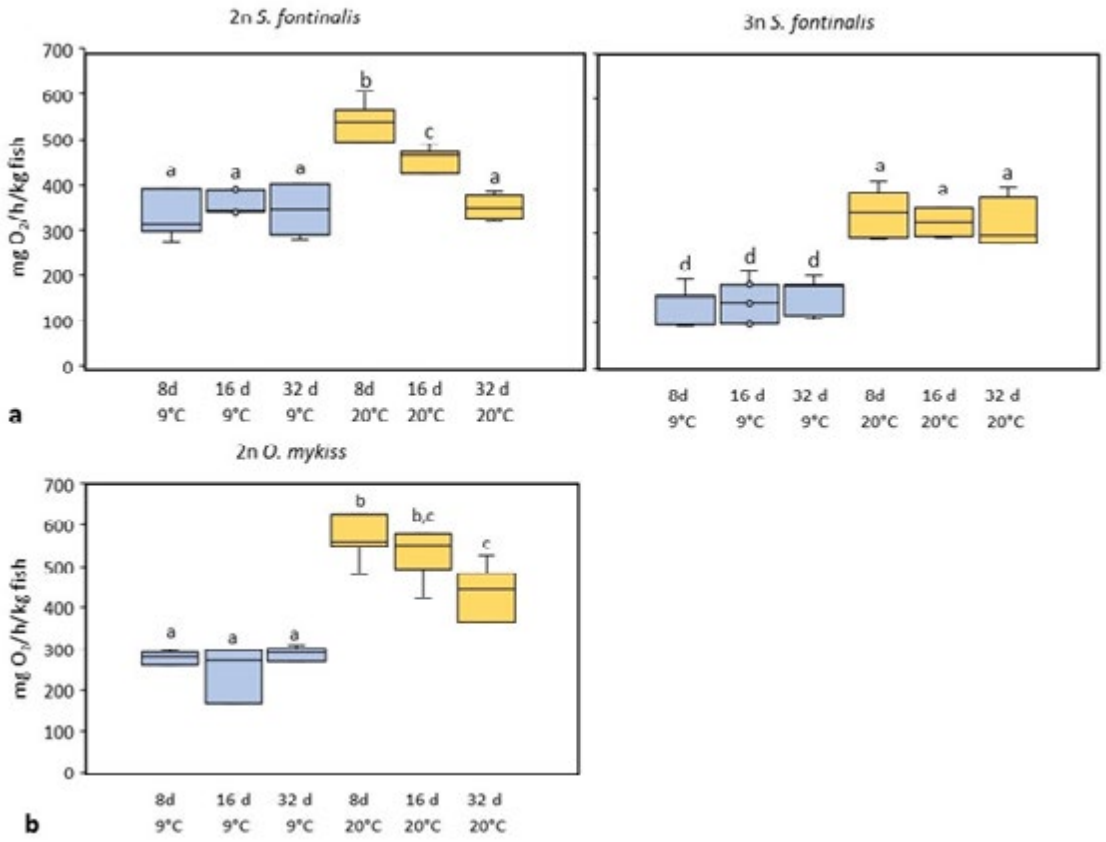

Figure 2: Boxplots of routine metabolic rate of 2n and 3n *S. fontinalis* (a, b) and 2n *O. mykiss* (c) after exposure to 20°C. Blue boxes: 9°C, orange boxes: 20°C. Data (n = 10) were compared between all species/ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test).

Differences between Fed and Starving Fish (Fig. 3)

In 2n *S. fontinalis* RMR differed significantly between fed and starving fish. At 9°C it was decreased in starving fish in comparison to fed ones, at 20°C it was increased. RMR of 3n *S. fontinalis* did not differ ($P > 0.05$) between fed and starving fish neither at 9°C nor at 20°C.

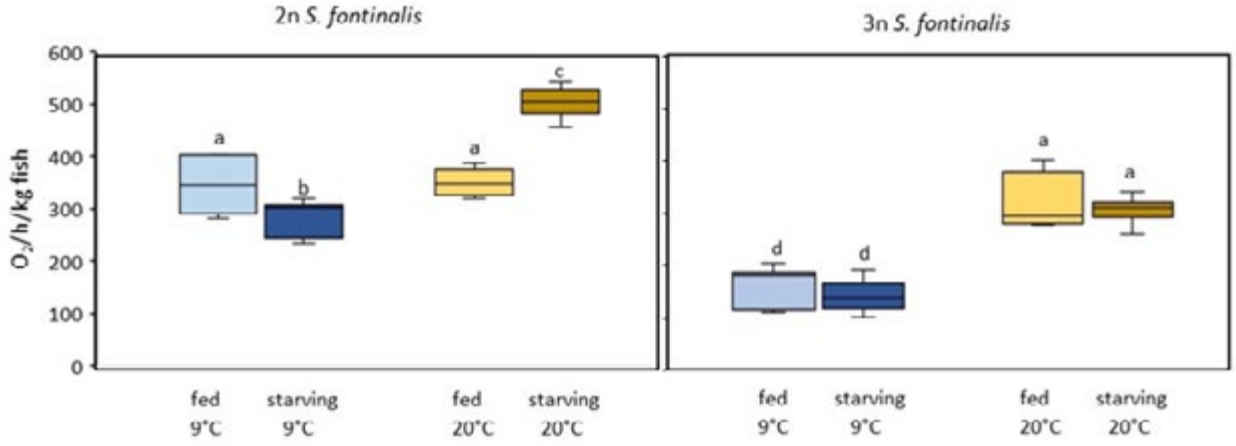

Figure 3: Routine metabolic rate of fed and 5d starving 2n and 3n *S. fontinalis* acclimated to 9°C and exposed to 20°C. Light blue boxes: fed fish at 9°C, dark blue boxes: starving fish at 9°C, light orange boxes: fed fish at 20°C, dark orange boxes: starving fish at 20°C. Data (n = 10) were compared between all ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test)

Particular and Dissolved Excretion

Differences in Particular Excretion between 9°C and 20°C (Fig. 4)

Dry weight and phosphorus concentration of faeces did not differ between the species/ploidy levels and between the temperature regimes ($P > 0.05$). Protein concentration of faeces was similar in 2n and 3n *S. fontinalis* and in *O. mykiss* ($P > 0.05$) at 9°C. After 32 d exposure to 20°C protein concentration of faeces of 2n and 3n S. fontinalis was significantly ($P < 0.05$) lower than at 9°C, that of O. mykiss did not differ between 9°C and 20°C. The lipid concentration of faeces of 2n *S. fontinalis* and 2n *O. mykiss* acclimated to 9°C was similar, that of 3n *S. fontinalis* was significantly lower. At 20°C lipid content of faeces of 2n and 3 n *S. fontinalis* and of O. mykiss was significantly decreased in comparison to 9°C.

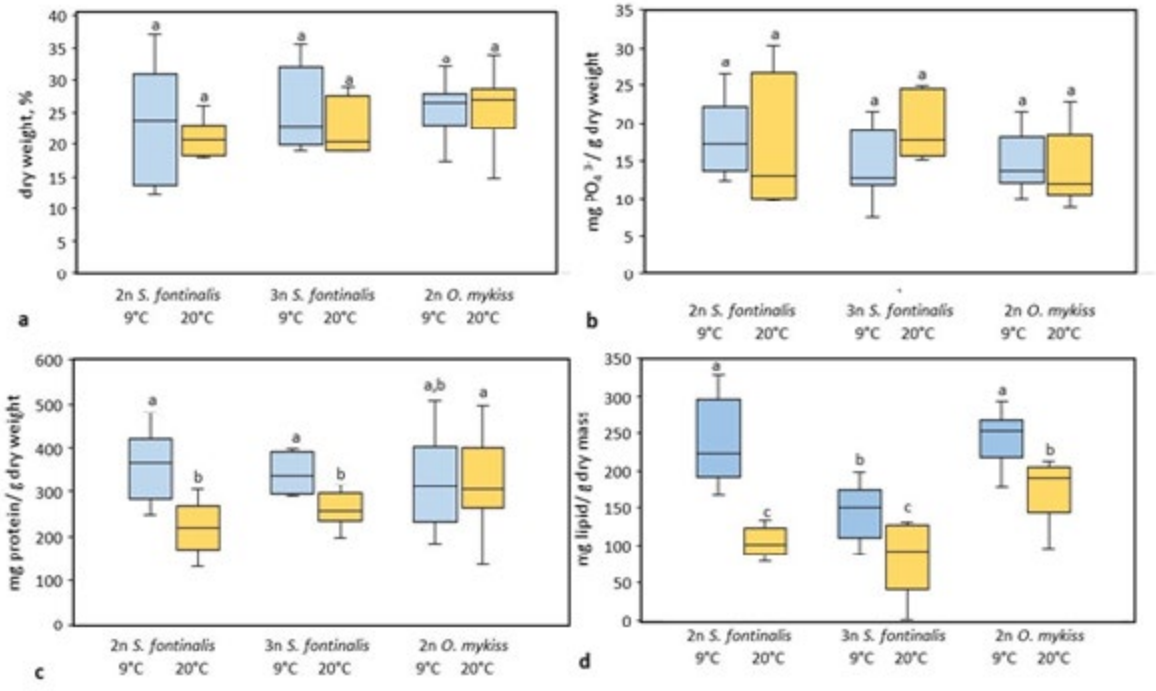

Figure 4: Faeces dry weight (a) and phosphate (b), protein (c), and lipid (d) content of 2n and 3n *S. fontinalis* and *O. mykiss* acclimated to 9°C (blue boxes) and exposed to 20°C for 32d (orange boxes). Data (n = 20) were compared between all species/ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test).

Differences in Dissolved Excretion between 9°C and 20°C (Fig. 5)

NH4-N, total nitrogen bound (tNB), and o-PO_4-P concentration of ground water were < 0.005 mg l-1. The quantities of NH_4-N, t-ON-N, and o-PO4-P excreted into the water did not differ ($P > 0.05$) between the species/ploidy levels and between the temperature regimes.

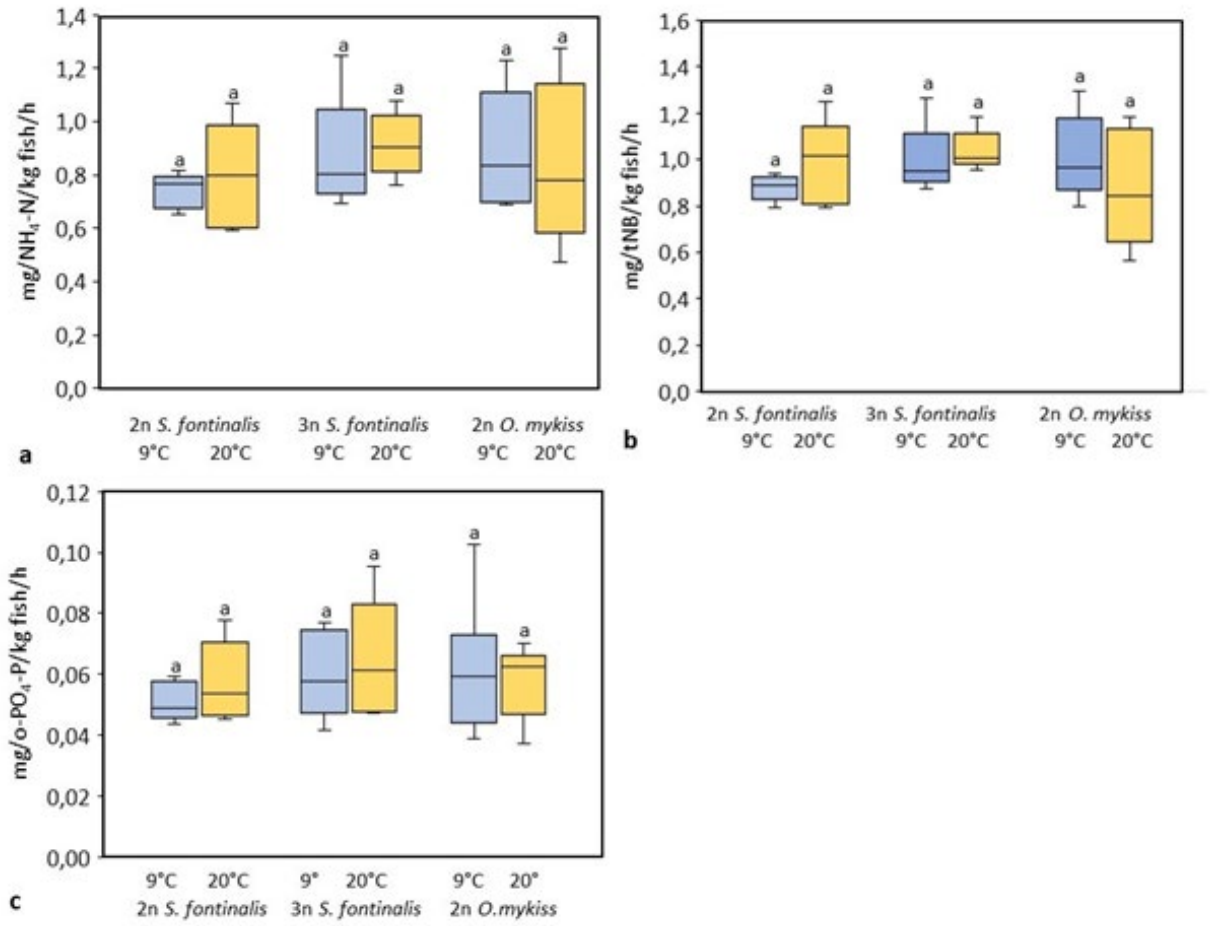

Figure 5: Ammonium (NH4-N [a], tNB [total nitrogen bound] [b], and orthophosphate (c) excretion of 2n and 3n *S. fontinalis* and of *O. mykiss* acclimated to 9°C (blue boxes) and exposed for 32d to 20°C (orange boxes). Data (n = 10) were compared between species/ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test).

Differences in Dissolved Excretion between Fed and Starving Fish (Fig. 6)

The quantities of NH_4-N, tNB, and o-PO_4-P excreted into the water were significantly ($P > 0.05$) decreased in starving 2n and 3n *S. fontinalis*. For the nitrogen compounds, the decrease was greater at 9°C than at 20°C. This was similar for both ploidy levels.

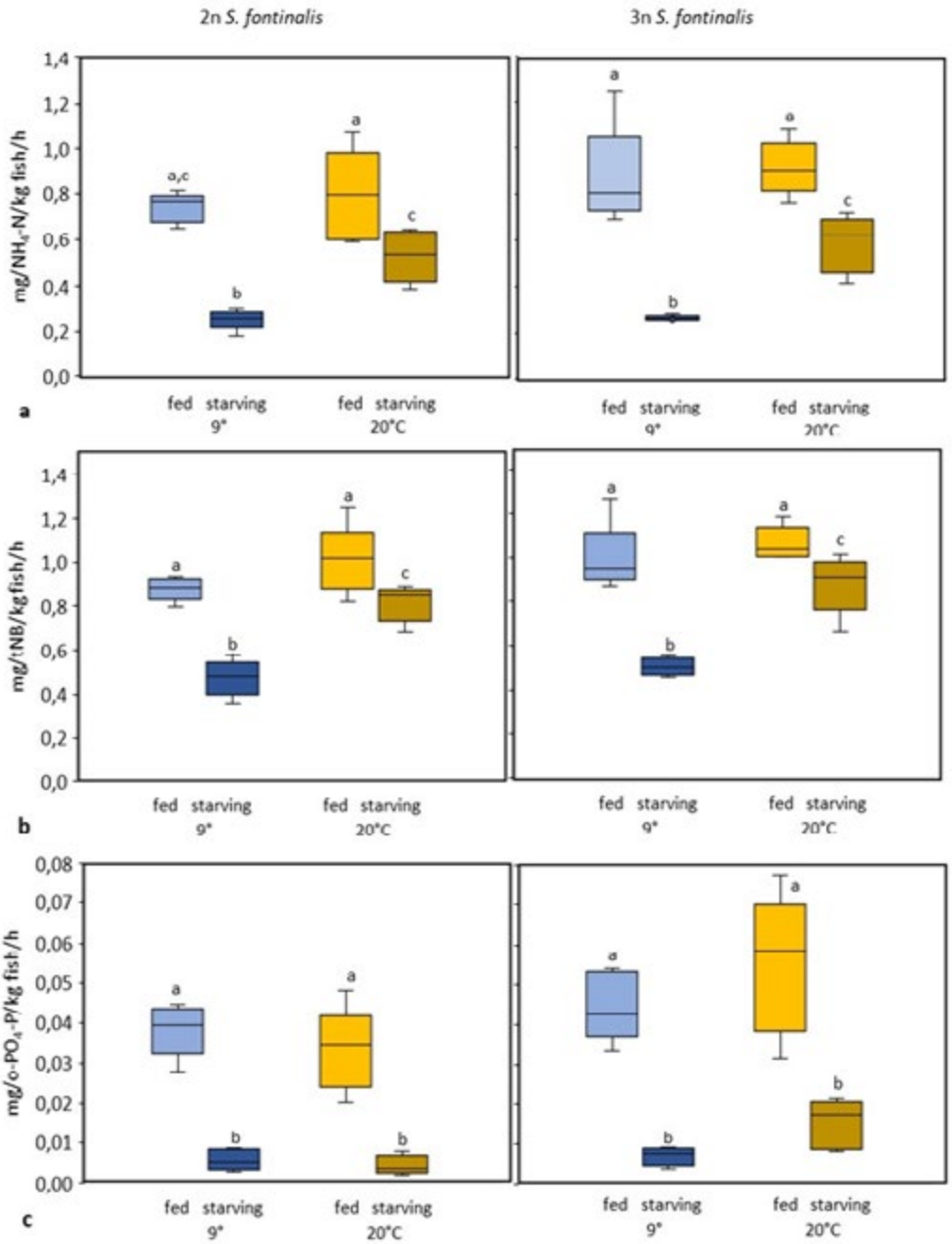

Figure 6: Ammonium (NH4-N [a]), tNB (total nitrogen bound [b]), and orthophosphate (c) excretion of fed and 5d starving 2n and 3n *S. fontinalis* acclimated to 9°C and exposed to 20°C. Light blue boxes: fed fish at 9°C, dark blue boxes: starving fish at 9°C, light orange boxes: fed fish at 20°C, dark orange boxes: starving fish at 20°C. Data (n = 10) were compared between all ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test).

Fish Energy Reserves and Cell Energy Status Differences between 9°C and 20°C (Fig. 7)

Liver glycogen, triglycerides and ATP concentration and adenylate energy charge, and visceral fat concentration did not differ between 2n and 3n *S. fontinalis* and 2n *O. mykiss* acclimated to 9°C. In fish exposed to 20°C for 32 d liver glycogen and visceral fat were significantly ($P < 0.05$) decreased, while liver triglycerides were not affected. Liver ATP levels of 2n and 3n *S. fontinalis* did not differ between 9°C and 20°C, ATP levels of *O. mykiss* were significantly increased at 20°C. Liver adenylate energy charge, muscle glycogen concentration (data not shown) and muscle lipid concentration (data not shown) did not differ between 9°C and 20°C in all investigated species and ploidy levels.

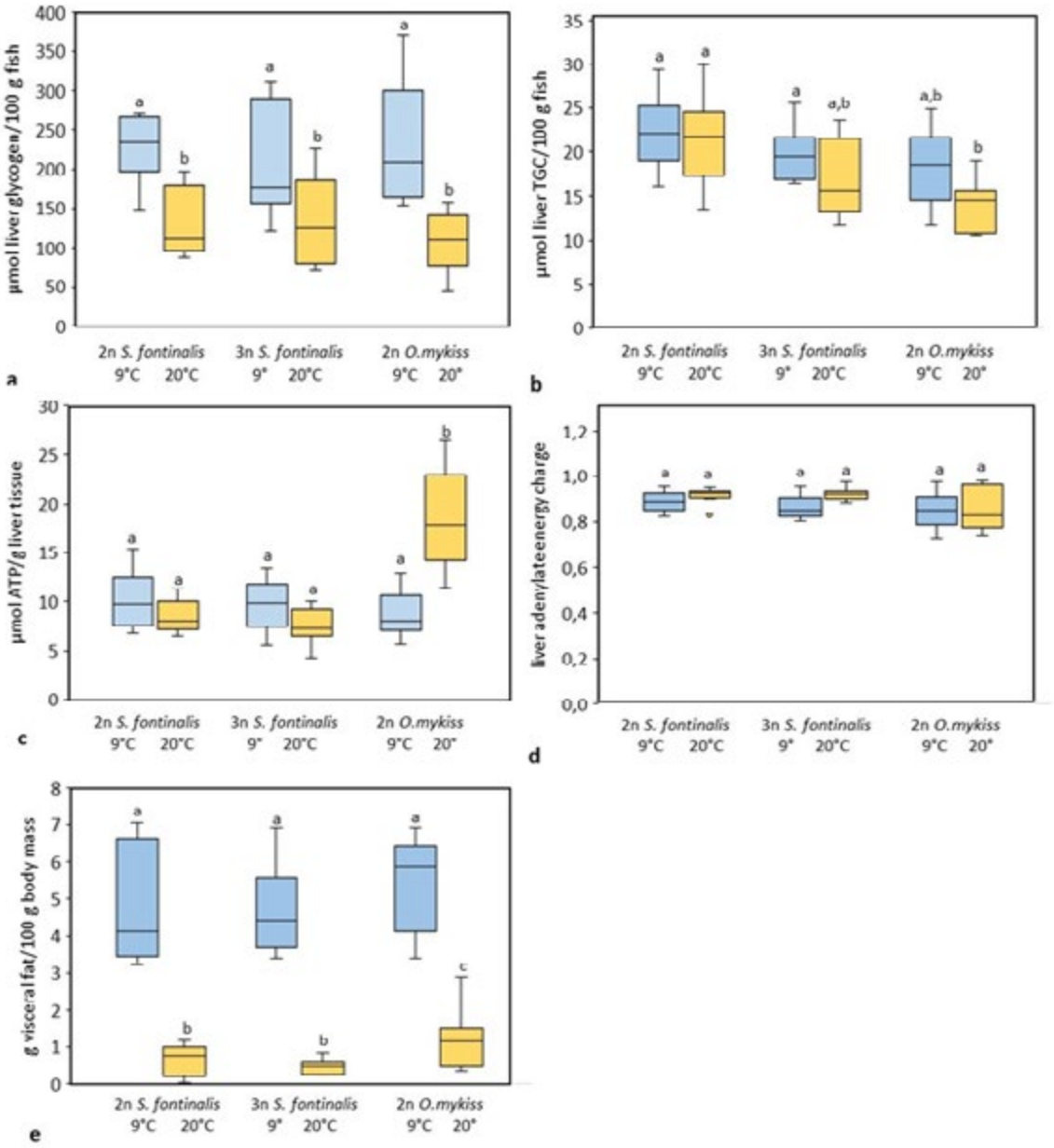

Figure 7: Liver glycogen (a), triglycerides "(TGC)" (b), ATP (c), adenylate energy charge (d), and visceral fat (e) of 2n and 3n *S. fontinalis* and *O. mykiss* acclimated to 9°C (blue boxes) and exposed for 32d to 20°C (orange boxes). Data (n = 20) were compared between all species/ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test)

Differences between fed and starving fish (Figs. 8, 9)

Liver ATP concentration, liver adenylate energy charge, and muscle glycogen concentration were similar ($P > 0.05$) in fed and starving 2n and 3n, *S. fontinalis* acclimated to 9°C and exposed to 20°C. Liver glycogen and triglycerides concentrations and muscle lipid concentration were similar between fed and starving 2n and 3n *S. fontinalis* at 9°C. At 20°C, liver glycogen and triglycerides were significantly decreased in starving fish in comparison to fed ones ($P < 0.05$). Muscle lipid concentration showed a non-significant decrease in starving fish. Visceral fat was significantly decreased in starving 2n and 3n *S. fontinalis* at 9°C. At 20°C, no significant differences were detectable between fed and starving fish.

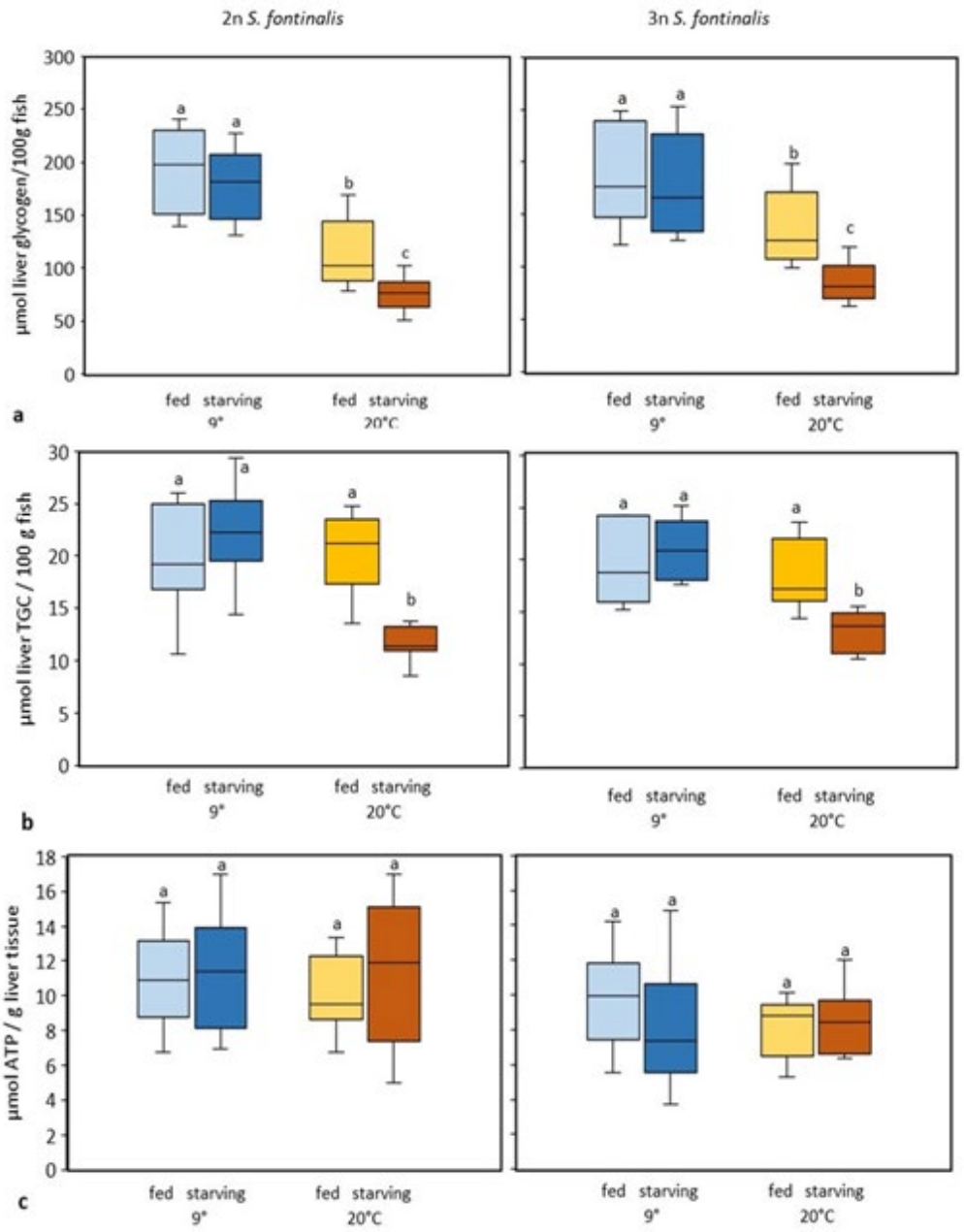

Figure 8: Liver glycogen (a), triglycerides (TGC) (b), and ATP concentrations (c) of fed and 5d starving 2n and 3n *S. fontinalis* acclimated to 9°C and exposed to 20°C. Light blue boxes: fed fish at 9°C, dark blue boxes: starving fish at 9°C, light orange boxes: fed fish at 20°C, dark orange boxes: starving fish at 20°C. Data (n = 20) were compared between all ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test)

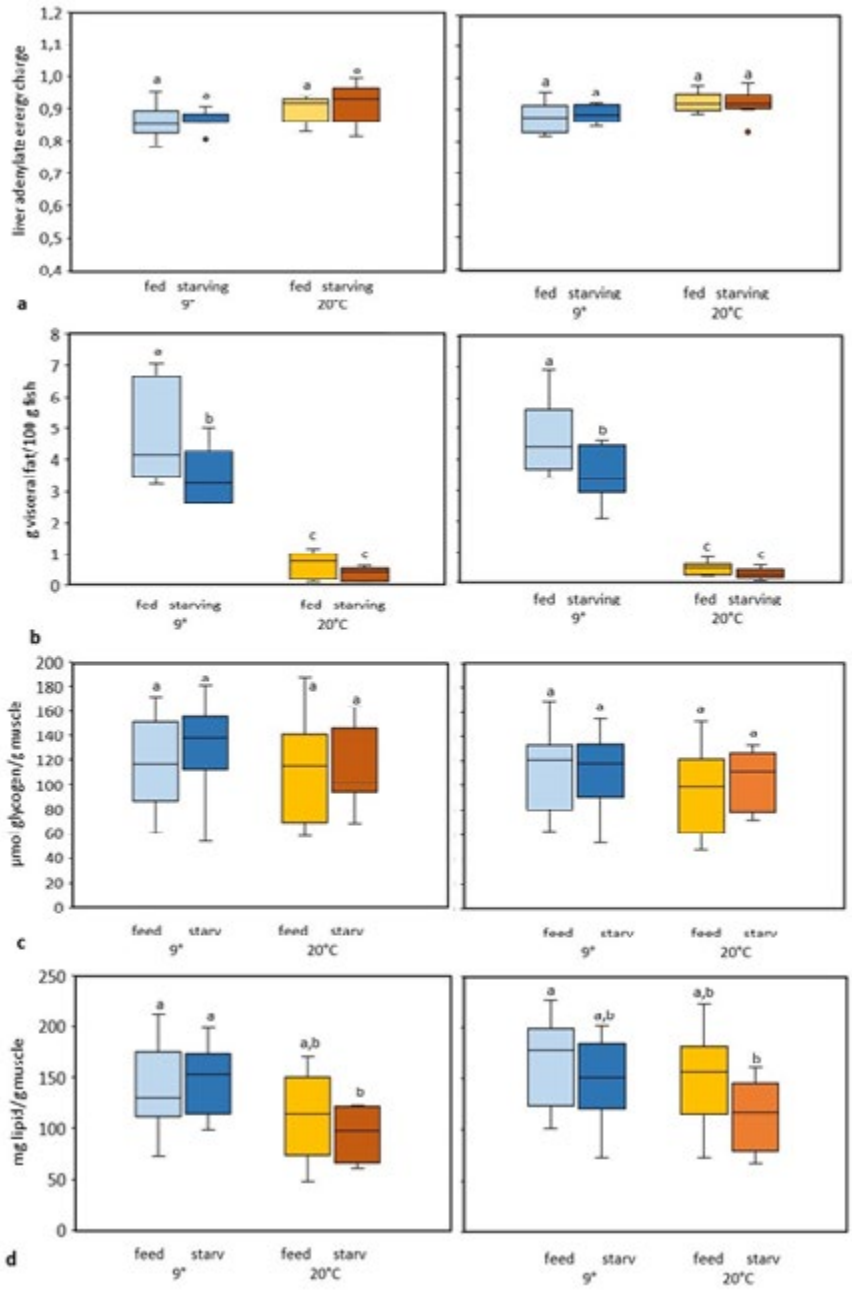

Figure 9: Liver adenylate energy charge (a), visceral fat (b), muscle glycogen (c), and muscle lipid concentration (d) of fed and 5d starving 2n and 3n *z`* acclimated to 9°C and exposed to 20°C. Light blue boxes: fed fish at 9°C, dark blue boxes: starving fish at 9°C, light orange boxes: fed fish at 20°C, dark orange boxes: starving fish at 20°C. Data (n = 20) were compared between all ploidy levels and temperatures. Those superscripted by different letters are significantly different (ANOVA with subsequent Tukey posthoc test).

Discussion

Routine Metabolic Rate (RMR)

Different types of metabolic thermal compensation occurred in the investigated Salmonidae when exposed to 20°C for 32 d. In 2n, *S. fontinalis* RMR was re-regulated to values similar to acclimation temperature that represents a total metabolic thermal compensation. Therefore, it can be concluded that 2n *S. fontinalis* is able of adjusting to a wide range of thermal regimes, which is conform to previous studies [7, 50]. Also, in *O. mykiss* metabolic thermal compensation occurred. However, it was only partial as RMR of fish exposed to 20°C remained 50 % over the values measured at 9°C. 3n *S. fontinalis* had lower RMR than 2n *S. fontinalis* under acclimation conditions. This is in accordance to previous data [51, 52]. During a 32d exposure to 20°C 3n *S. fontinalis* did not re-regulate the RMR to acclimatization conditions, as it was constantly increased for > 110%. This could indicate decreased thermal plasticity of 3n S. fontinalis. Inability of 3n *S. fontinalis* for metabolic thermal compensation did not influence performance of fish at 20°C, as the equilibrium rate was 100% and the growth rate was higher than at 9°C. "One study [53] found no ploidy specific effect on critical thermal maximum. According to another study [54] 3n *S. fontinalis* had higher metabolic rates than diploids at lower temperature, and lower metabolic rates than diploids at higher temperature and it was suggested that this fact might lead to lower thermal tolerance in triploids.

RMR of 5-day starving 2n *S. fontinalis* was decreased in comparison to fed ones at acclimation conditions. In teleost fish, a decrease of the metabolic rate in response to food withdrawal is a mechanism to preserve energy reserves [4, 55 and 56]. At acclimation temperature, standard metabolic rate was reduced due to starving in different species [57-61]. However, RMR of 2n *S. fontinalis* exposed to 20°C increased due to starvation. This might indicate the activation of oxidative metabolism to generate sufficient energy to maintain cell homeostasis. RMR of 3n *S. fontinalis* did not respond to a 5-day starving period, neither at 9°C nor at 20°C. This could be interpreted as an inflexibility of 3 n *S. fontinalis* to respond to changing physiological situations. The present data demonstrate that the concept of metabolic rate reduction due to food deprivation cannot be generalized but depends on the ploidy level and on the temperature. Therefore, also the common aquaculture practice of food withdrawal at elevated temperature to counteract oxygen depletion might be only of limited value.

Particular and Dissolved Excretion

In 2n and 3n *S. fontinalis* and in 2n *O. mykiss* exposure to 20°C had no negative on the composition of faeces and the quantities of excreted nitrogen and phosphate. Faeces dry weight did not differ between fish acclimated to 9°C and exposed to 20°C for 32 d. This is an indication that water absorption rate in the intestine was similar for the two temperature regimes and that faeces consistence was not affected. In addition, phosphorus concentration of faeces and the quantities of excreted dissolved phosphorus did not differ between 9°C and 20°C. This is conformed to an earlier study on *O. mykiss* where phosphorus waste outputs were similar in a temperature range from 6 -15°C [26]. In 2n and 3n *S. fontinalis* exposed to 20°C the protein and lipid concentration of faeces was lower than at 9°, in *O mykiss* only the lipid concentration indicating better feed digestibility at elevated temperature. The data on 2n *S. fontinalis* agree to those of [7]. A positive relationship between nutritient digestibility and temperature has also been observed in studies on S. salar [62-63] while other studies noted no effect [64-66]. Discrepancies between the cited studies may be related to the tested temperature range. Digestibility depends on gut transit time and activities of digestive enzymes [67-69]. Gut transit time decreases with temperature while temperature optima of digestive enzymes are type and species-specific [70]. The excretion of dissolved ammonium (NH4-N) and total nitrogen bound did not differ between 2n and 3n *S. fontinalis* and 2n *O. mykiss* acclimated to 9°C and exposed to 20°C for 32d. Increased ammonium excretion would be an indication for increased protein catabolism either due to higher amount of feed uptake or due to catabolism of muscle tissue [19]. In addition, other studies on *O. mykiss* found no differences in ammonium excretion in relation to temperature [26, 27]. Under practical considerations the present data demonstrate, that excretion load of water was not increased by elevated temperature and that the species and ploidy specific differences were only minor. A 5-d starving period decreased the quantities of excreted dissolved ammonium, total nitrogen bound, and phosphate in both ploidy levels of S. fontinalis and at both tested temperature regimes. In starving fish, the excretion of ammonium and total nitrogen bound was higher at 20°C than at 9°C which might be an indication for increased metabolic activity and probably also for a higher rate of protein catabolism at elevated temperature. The data are in contrast to previous ones demonstrating a positive relation between ammonium excretion of non-fed teleost fish species and temperature [21-24]. Differences may be due to the tested temperature and exposure time and the period of starving [71].

Fish Energy Reserves and Cell Energy Status

Exposure to 20°C was an energetic stress for 2n and 3n *S. fontinalis* and 2n *O. mykiss* as the organismic energy reserves (visceral body fat, liver glycogen) were decreased. Perivisceral lipid is a main energy depot located around the digestive tract [72]. Glycogen and triglycerides are the main hepatic storage forms of energy [73]. Exhaustion of energy levels might impede a response to additional stress factors. Cellular ATP concentration and cellular adenylate energy charge were not affected by temperature indicating that cell energy status and homeostasis could be maintained. Ploidy and species-specific differences in energy status were only minor. Liver glycogen depletion in response to thermal stress has been described in previous studies for [74, 75]. Muscles glycogen concentration remained at constant levels. Muscle glycogen is essential for its short-term energy supply. Muscle function is seriously compromised when the glycogen stores are reduced to low levels [76].

Responses of energy metabolism in relation to starving were tem-

perature dependent. At 9°C, starving 2n and 3n *S. fontinalis* used visceral lipids as energy resources as their amount significantly decreased. This is conformed to previous studies [77, 78]. At 20°C, for both ploidy levels liver glycogen and triglycerides decreased significantly and muscle lipids non-significantly demonstrating their use as energy resources. This is probably due to the fact, that visceral fat depots were already depleted before the starving period began. Therefore, food deprivation at elevated water temperature should be considered critical. It can lead to complete exhaustion of energy resources and to a loss of cell homeostasis. In addition, it was that lipid catabolism was high during the initial period of starving and was followed by carbohydrate utilization when lipid reserves were depleted [71]. In the present study there was no indication for metabolization of muscle protein, as body mass of fed and starving fish did not reveal significant differences and ammonium excretion was not increased [71].

Conclusions

In Salmonidae different types of metabolic thermal compensation occur after 32 d exposure to 20°C, a full thermal compensation in 2n *S. fontinalis*, a partial one in 2n *O. mykiss* and none at all in 3n *S. fontinalis.* When offering similar feed quantities, temperature related effects on excretion of waste products were minor for all investigated species and ploidy levels. Exposure to 20°C represented an energetic stress situation for 2n and 3n *S. fontinalis* and 2n *O. mykiss* as the visceral fat and liver glycogen depots were depleted. The temperature related effects of a 5-d starving period were investigated in 2n and 3n *S. fontinalis* at the end of the 32d lasting experiment. The effect on the metabolic rate was temperature and ploidy level specific (2n *S. fontinalis:* decrease at 9°C, increase at 20°C; 3n S. fontinalis: no changes). After 5-d starvation, quantities of excreted dissolved nitrogen and phosphorus were decreased in comparison to fed fish at 9°C and 20°C. At elevated temperature, starving can result in a critical energetic stress situation which might lead to complete exhaustion of energy resources.

Supplementary Information

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Authors' contributions

As sole author F. Lahnsteiner designed the study, performed the experiments and analysis. He wrote the manuscript text and prepared the figures. He reviewed the manuscript and approved the final version.

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Availability of Data and Material

The author declares that all data supporting the findings of this study are included within the article. The datasets of the current study are available on reasonable request.

Declarations

Ethics Approval and Consent to Participate

The project was evaluated and approved by a committee of the Federal Agency for Water Management and of the Federal Ministry for Agriculture, Regions and Tourism. It was classified as project "exposing fish to conditions not significantly exceeding stress situations occurring in fish farms". It is officially listed as institutional project 3143.

Human and Animal Ethics

Experiments were carried out in accordance with Austrian regulations governing animal welfare and protection (Tierversuchsgesetz, BGBl. I Nr. 114/2012) and with the EU directive 2010/63/EU for animal experiments. All practices and procedures for the care and management of animals based on current best practice under the supervision of skilled workers of the fish farm Kreuzstein and of the responsible veterinarian.

Consent for Publication

Not applicable

Competing Interests

The author declares that he has no competing interests.

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