Overwinter fasting and re-feeding in rainbow trout: Plasma growth hormone and cortisol levels in relation to energy mobilization

T. G. Pottinger[a]*, M. Rand-Weaver[b] and J. P. Sumpter[b]

[a] *The NERC Centre for Ecology and Hydrology, Windermere Laboratory, Far Sawrey, Ambleside, Cumbria LA22 0LP, U.K.*

[b] *Department of Biology and Biochemistry, Brunel University, Uxbridge, Middlesex, UB8 3PH, U.K.*

* Corresponding author. Tel.: +44 (0)15394 42468; Fax: +44 (0)15394 46914;

E-mail address: tgp@ceh.ac.uk

Abstract

This study investigated the roles of cortisol and growth hormone during a period of fasting in overwintering salmonid fish. Indices of carbohydrate (plasma glucose, liver glycogen), lipid (plasma free fatty acids) and protein metabolism (plasma protein, total plasma amino acids) were determined, together with plasma growth hormone (GH), cortisol and somatolactin levels (SL) at intervals in three groups of rainbow trout (continuously fed; fasted for 9 weeks then fed; fasted for 17 weeks). In fasted fish, a decline in body weight and condition factor was accompanied by reduced plasma glucose and hepatic glycogen and increased plasma FFA. No consistent elevation of plasma GH occurred until after 8 weeks of fasting when plasma GH levels increased nine-fold. No changes were observed in plasma total protein and AA until between weeks 13 and 17 when both were reduced significantly. When previously fasted fish resumed feeding, plasma glucose and FFA, and hepatic glycogen levels rapidly returned to control values and weight gain resumed. No significant changes in plasma cortisol levels, related to feeding regime, were evident at any point during the study and there was no evidence that SL played an active role in the response to fasting. The results suggest that overwinter fasting may not represent a significant nutritional stressor to rainbow trout and that energy mobilisation during fasting may be achieved without the involvement of GH, cortisol or SL.

Keywords: cortisol, fasting, free fatty acids, growth hormone, glucose, glycogen, Oncorhynchus mykiss, somatolactin.

1. Introduction

Salmonid fish in northern latitudes experience low winter water temperatures often coupled with limited food availability. Both behavioural and physiological strategies are employed to meet the energy requirements of the fish during such periods but they primarily rely upon stored fat. The extent to which reserves are sequestered depends upon many variables including the size of the fish, reproductive history, availability and type of food, and thermal environment (Ultsch, 1989; Metcalfe and Thorpe, 1992; Hutchings et al., 1999). The mobilisation of energy reserves under conditions of nutritional stress is under endocrine control.

As is the case for other vertebrates, the hormonal regulation of metabolism in fish is a complex multifactorial process involving insulin and insulin-like growth factor (IGF-1; Plisetskaya, 1995; Duan, 1997; Moriyama et al., 2000), glucagon (Plisetskaya and Mommsen, 1996; Moon, 1998), glucagon-like peptide-1 (Mommsen, 2000), growth hormone (GH; Bjornsson, 1997), catecholamines (Fabbri et al., 1998), thyroid hormone (Eales, 1988), cortisol (Mommsen et al., 1999) and possibly somatolactin (SL; Rand-Weaver et al.,1995; Company et al., 2001). There are several studies that address the roles of some of these hormones during periods of nutritional stress or anorexia in fish, but an overall consensus regarding the endocrine profile that characterises fasting and/or starvation is lacking. In particular, although both cortisol and GH are characterised in fish as multi-functional hormones with involvement in metabolic regulation their precise roles, particularly that of cortisol, during periods of fasting remain unclear.

Plasma levels of the glucocorticoids cortisol or corticosterone are consistently reported to be elevated in response to fasting or malnutrition in homeothermic species as diverse as rodents (Tang et al., 1984; Challet et al., 1995; van Haasteren et al., 1996; Chang et al., 2002;), humans

(Bergendahl et al., 1996; Katz et al., 1998; El-Migdadi et al., 2002), northern elephant seal pups (Ortiz et al., 2001) and penguins (Groscolas and Robin, 2001). In contrast, the evidence in fish is contradictory. Plasma cortisol levels in otherwise unstressed fish are variously reported to be unaffected by fasting (Arctic charr, *Salvelinus alpinus*: Joergensen et al., 1999; rainbow trout, *Oncorhynchus mykiss*: Sumpter et al., 1991; Holloway et al., 1994; Reddy et al., 1995; coho salmon, *O. kisutch*: Vijayan et al., 1993; plaice, *Pleuronectes platessa*: White and Fletcher, 1986), reduced by fasting (chinook salmon, *O. tshawytscha*: Barton et al., 1988; rainbow trout: Farbridge and Leatherland, 1992a) or increased by fasting (Arctic charr: Jorgensen et al., 2002; rainbow trout: Blom et al., 2000; goby, *Gillichthys mirabilis*: Kelley et al., 2001; coho salmon: Varnavsky et al., 1995). Notwithstanding the variation in experimental conditions between these studies there remains considerable uncertainty about the regulation of plasma cortisol under conditions of anorexia in fish, uncertainty that perhaps mirrors the lack of clarity regarding the precise metabolic role of cortisol in teleost fish (Mommsen et al., 1999).

For GH, the picture is less contradictory. In homeotherms it is well established that during fasting plasma GH levels rise. Under conditions of nutritional stress GH is concerned primarily with regulation of protein metabolism - both stimulation of protein synthesis and inhibition of protein breakdown (Norrelund et al., 2002). GH also promotes energy expenditure via increased lipolysis and ketogenesis and decreases insulin-mediated activation of the glycogen synthase in liver and muscle (Bak et al., 1991; Moller et al., 1995). GH is believed to possess similar functions in fish (Björnsson, 1997). The generally held view is that fasting and/or starvation result in an increase in circulating levels of GH, consequent to a reduced negative feedback by hepatic IGF-1 (Björnsson, 1997), arising from a decrease in the sensitivity of the liver to GH stimulation. However, there is some variation reported in this pattern, particularly regarding the

period of fasting required to elicit a rise in GH. Plasma GH levels are increased by fasting in a number of species of fish (rainbow trout: Sumpter et al., 1991; Takahashi et al., 1991; Farbridge and Leatherland, 1992a,b; Johnsson et al., 1996; coho salmon: Varnavsky et al., 1995; striped bass, *Morone saxatilis*: Small et al., 2002; tilapia, *Oreochromis mossambicus*: Weber and Grau, 1999) although this increase may be minimal and inconsistent (Holloway et al., 1994). Some results that diverge from this pattern have been reported. In rainbow trout fed a reduced ration (once weekly) GH levels were lower than in those fed more frequently (Farbridge et al., 1992).

An additional, but less well-characterised candidate for involvement in the metabolic response to fasting is the pituitary hormone somatolactin (SL). The precise role(s) of SL in fish have yet to be fully defined but some evidence suggests a metabolic function; plasma SL levels are reported to vary inversely with plasma GH levels (Rand-Weaver et al., 1995; Company et al., 2001) and SL has been suggested to function in the adaptation to fasting or reduced food intake (Mingarro et al., 2002). Our understanding of the status and function of the signal peptides leptin and ghrelin in fish is also limited. Leptins are implicated in signalling of energy status, and mediation of energy utilisation and mobilisation (Margetic et al., 2002). Although leptin has yet to be isolated from teleost fish, immunoreactivity to heterologous leptin antibodies has been detected in a number of fish species (Johnson et al., 2000) and limited data suggest that site of synthesis (Mustonen et al., 2002a) and mode of action (Londraville and Duvall, 2002; Mustonen et al., 2002b) may differ from that in mammals. Ghrelin is involved in GH release and energy regulation in mammals (Yoshihara et al., 2002). Data for fish are limited but a gene encoding a peptide with high homology to human ghrelin has been isolated in goldfish (*Carassius auratus*; Unniappan et al., 2002). Given that the focus of this study is to resolve uncertainty surrounding the regulation of cortisol and GH during fasting in fish neither leptin or ghrelin will be further

considered.

The intention of this study was to examine the profile of plasma cortisol and GH levels during a period of feed restriction in trout with a high degree of temporal resolution and concomitant measures of energy mobilisation. Plasma levels of GH, cortisol and SL were determined in the blood of rainbow trout during a 120 day overwintering period. Three nutritional conditions were imposed on the fish; (i) normal rations; (ii) fasting followed by refeeding; and (iii) continuous fasting. Representative indicators of metabolic condition were also determined (plasma glucose, total protein, free fatty acids and total amino acids and liver glycogen concentrations) over the same time course to inform our understanding of the links between availability of substrates to tissues and endocrine status.

2. Materials and methods

2.1 Experimental conditions

Nine hundred, two-year-old, all-female rainbow trout (Isle-of-Man strain, mean weight □ SE at start of experiment: 282.9 □ 9.7 g, n = 60) were evenly and randomly distributed between six circular, 1000 l, glass fibre holding tanks. Each tank was supplied with a constant flow of lake water (30 l min^{-1}) at ambient temperature. The variation in water temperature during the experimental period is depicted in Fig. 1a. The fish were allowed to acclimate to these conditions for 1 month, during which time they were fed once daily, 5 times per week with commercial feed (BP Mainstream) at the manufacturers recommended rate (1% – 2% body weight day^{-1}).

On day 0 of the experimental period (22 February), a random sample of ten fish was netted from the first tank with the minimum of disturbance to the remaining fish. The fish were anaesthetised

(2-phenoxyethanol, 1:2000) and a blood sample was rapidly removed from each fish, via the caudal vessels, into heparinized syringes. Blood sampling of each group of ten fish was completed within 5 min. Blood was transferred to capped centrifuge tubes and placed on ice. The fish were killed by a blow to the head after which body weight and fork length were recorded. Livers were removed from the first five fish, and frozen immediately in liquid nitrogen. This entire process was repeated for each of the remaining five tanks. Blood was centrifuged and plasma was transferred to capped tubes and stored at -20^{o}C until required for assay. Sampling was always carried out in the morning, between 09.00h and 12.00h.

The six experimental tanks were randomly allocated numbers 1 to 6. From day 0 onwards, food was withheld from tanks 3 to 6. Further samples of fish were taken on days 3, 7, 16, 29 and 58. On day 63 feeding was resumed for tanks 3 and 4 and these tanks only were sampled on days 64 and 66. The digestive tracts of these fish were inspected to ensure that feeding had recommenced. Additional samples were taken from all six tanks on days 71, 78, 92 and 120.

2.2 Measurement of plasma GH, cortisol and SL

Plasma GH was measured using a specific radioimmunoassay developed for recombinant trout GH (Eurogentec Standard Operating Procedure 1013). Plasma samples (50µl) were incubated with anti-trout GH serum (1:750,000, 100µl) in the presence of labelled trout GH (20,000 cpm, 100µl) for 16h. Anti-rabbit IgG precipitating solution (UCB Bioproducts, Brussels, Belgium) was then added, and the tubes incubated for a further 30 min before centrifugation to separate bound and free GH. The inter- and intra-assay variations were 6% and 4% respectively. Plasma SL levels were determined as described previously (Rand-Weaver *et al*., 1992). Plasma samples (50µl) were incubated with anti-coho salmon SL serum (1:20,000, 50µl) for 72h after which ^{125}I-

labelled coho salmon SL (5000 cpm, 50µl) was added. Following a further 24h incubation, goat anti-rabbit IgG (1:40, 50µl) was added. Bound and free label were separated by centrifugation, and the pellets counted in a γ-counter. Inter- and intra-assay variations were 9.3 and 4.9% respectively. Plasma cortisol levels were determined in fish 1, 2, 9 and 10 from every batch of 10 using a previously validated cortisol radioimmunoassay (Pickering et al., 1987).

2.3 Measurement of glucose, protein, glycogen, and total amino acids

Plasma glucose levels were quantified using the glucose oxidase method (Sigma Diagnostics, procedure no. 510-A). Liver glycogen was determined according to the method of Roehrig and Allred (1974), plasma protein was determined according to the method of Ohnishi and Barr (1978) and total plasma amino acids were assayed as described by Rosen (1957). Plasma free fatty acids were measured using the WAKO NEFA-C kit (Alpha Labs. Ltd, Eastleigh, UK). Plasma glucose and FFA's were assayed in all samples. Plasma amino acids and protein, and liver glycogen were determined on days 29 (prior to re-feeding), 71, 92 and 120 only.

2.4 Statistical analysis

The effects of the experimental procedure on endocrine, metabolic and somatic data were assessed by two-way analysis of variance (ANOVA, Genstat) with time (day of sampling) and treatment (fed, re-fed, fasted) as factors. The cortisol, SL, GH, FFA, glucose and glycogen data were log transformed to improve homogeneity of variance. Multiple comparison post tests to assess significant differences between times or treatment groups were carried out using the estimated standard error of the differences between means provided by the Genstat output.

3. Results

Water temperature increased in a linear fashion with time during the experimental period from a minimum of 5ºC to a maximum of 16ºC (Fig. 1a.).

[insert Fig. 1]

3.1 Weight

There was no significant difference in weight between the three experimental groups at the start of the experiment (fed: 283 ± 17 g; re-fed: 280 ± 13 g; fasted: 286 ± 21 g; mean ± SEM, n=20; Fig 1b) but within 29 days there was a significant (P<0.001) decline in weight in both fasted groups (231 ± 13 g, 232 ± 10 g) relative to the fed fish (299 ± 17 g). No significant weight gain was evident in the fed group until day 78. In the re-fed fish, a significant increase in body weight was apparent within 8 days of the resumption of feeding (day 71). The body weight of the fasted fish on day 120 (219 ± 12 g) was significantly less than that on day 0 (P<0.001; 286 ± 21 g). At the end of the experimental period (day 120) significant differences in body weight remained between all three groups (P<0.001; fed: 530 ± 31 g; re-fed: 412 ± 20 g; fasted: 219 ± 12 g).

3.2 Co-efficient of condition

There was a significant (P<0.05, P<0.001) decline in condition factor [coefficient of condition; (100 x weight)/length^3] in both fasted groups within 14 days of the cessation of feeding (Fig. 1c) whereas condition factor in the fed group remained unchanged from day 0 until increasing significantly (P<0.01) between days 58 and day 71. Condition factor in the continuously fasted group declined significantly (P < 0.001) throughout the experimental period. In the re-fed group condition factor increased significantly (P<0.01) within 3 days of the onset of feeding (day 66: 1.123 ± 0.02) relative to the pre-feeding value (day 58: 1.045 ± 0.02) and was not significantly different from that of the control fish at the end of the experimental period.

3.3 Plasma cortisol

Overall, there was a small but statistically significant (P=0.039) effect of treatment on plasma cortisol levels with higher levels in fed fish (8.8 ± 1.0 ng ml^{-1}, n =88; Fig. 2a) and re-fed fish (7.9 ± 0.9 ng ml^{-1}) than fasted fish (5.9 ± 0.9 ng ml^{-1}). However, mean plasma cortisol levels displayed no consistent trend within any treatment group and the overall difference between treatments appears to be associated primarily with variation in mean plasma cortisol levels in the fed and re-fed groups during the first 14 days of the study. There was no significant difference between cortisol levels in the fasted group on day 0 (4.3 ± 1.8 ng ml^{-1}, n = 8) and on day 120 (10.9 ± 8.2 ng ml^{-1}).

[Insert Fig. 2]

3.4 Plasma GH

Within 29 days of the cessation of feeding, plasma GH levels in the re-fed group (prior to re-feeding) were significantly ($p<0.001$) elevated above levels in the control fish (2.17 ± 0.6 ng ml^{-1} cf. 0.54 ± 0.18 ng ml^{-1}; Fig. 2b) but within 14 days of re-feeding (day 78), plasma GH in these fish had declined to levels indistinguishable from controls. Plasma GH levels in the fasted fish, although slightly but significantly higher ($p<0.05$) than controls at day 16, did not rise above control values again until between days 58 and 71, after which a linear rise in plasma GH levels was observed in the fasted fish. Circulating GH levels in the fasted fish at the end of the experimental period (17.98 ± 3.2 ng ml^{-1}) were more than 18-fold higher than levels in the two fed groups (0.39 ± 0.1, 0.92 ± 0.3 ng ml^{-1}; $P<0.001$).

3.5 Plasma somatolactin

Between days 0 and 14, there was a highly significant ($p<0.001$), elevation of plasma SL levels in

the control group. Within 30 days of the cessation of feeding, plasma SL levels in one of the fasted groups of fish were significantly ($p<0.05$) lower than in either the second group of fasted fish, or the control fish (4.6 ± 0.8 cf. 6.8 ± 0.9, 6.4 ± 0.7 ng ml^{-1}; Fig. 2c). Somatolactin levels in one group of fasted fish remained significantly lower than levels in either of the other treatments groups throughout the experimental period. Between days 56 and 76 there was a significant ($p<0.001$) elevation of SL levels in both fed and re-fed groups of fish. At no point after day 10 did plasma SL levels in the re-fed group diverge significantly from levels in the control group of fish.

3.6 Metabolites

3.6.1 Plasma free fatty acids

Overall, there was a very pronounced ($P<0.001$; Fig. 3a) decline in mean plasma free fatty acid (FFA) levels in fed fish from day 0 (325.9 ± 36.4 μmol l^{-1}) through to day 78 (64.8 ± 8.2 μmol l^{-1}) after which FFA levels significantly increased to reach 301.3 ± 13.9 μmol l^{-1} at the end of the experiment, a level statistically indistinguishable from that at the start of the experiment. In both re-fed and fasted groups plasma FFA levels were significantly ($p<0.05$) elevated relative to the fed controls within 7 days of the cessation of feeding (341 ± 32, 333 ± 24 cf. 232 ± 29 μmols l^{-1}). This difference was increased upon and sustained until re-feeding on day 63 elicited a rapid drop in mean FFA levels in the re-fed fish from 332 ± 24 μmols l^{-1} on day 58 to 29 ± 5 μmols l^{-1} on day 71, slightly, but significantly, lower than the control (53.9 ± 5 μmols l^{-1}). Mean FFA levels in the re-fed groups rose between days 78 and 92 to reach levels indistinguishable from those at the start of the experiment by day 120 (255 ± 20 μmols l^{-1}). Mean plasma FFA levels declined steadily in the fasted fish between day 29 (464 ± 27 μmol l^{-1}) and day 120 (141 ± 18 μmol l^{-1}).

[Insert Fig. 3]

3.6.2 Plasma glucose

During the first 7 days of the study there was a transient ($p<0.001$) increase in glucose levels in control fish but within 9 days levels were again similar to those on day 0 (~ 150 mg 100 ml^{-1}; Fig. 3b). A further significant increase in plasma glucose levels in the fed fish occurred between days 71 and 92 ($P < 0.001$) before levels declined precipitously between day 92 and day 120, from 222 ± 11 mg 100 ml^{-1} to 74 ± 5 mg 100 ml^{-1} ($P<0.001$). Plasma glucose levels in the fasted (71.8 ± 3.7 mg 100 ml^{-1}) and re-fed (72.8 ± 1.9 mg 100 ml^{-1}) groups fell to levels significantly lower ($p<0.001$) than those of the controls (162.7 ± 9 mg 100 ml^{-1}) within 16 days of the cessation of feeding. Plasma glucose levels in the re-fed fish rose rapidly after the resumption of feeding on day 63 to reach levels indistinguishable from the controls within 8 days. No differences were detected between glucose levels in re-fed fish and control fish between day 71 and day 120. Plasma glucose levels in the fasted fish remained significantly lower than levels in the fed fish between days 16 and 120.

3.6.3 Plasma total protein

There were no significant differences in plasma total protein between treatment groups from day 29 to day 71 although levels increased significantly with time during this period from 74.7 ± 2.1 mg 100 ml^{-1} (overall mean; n = 60) to 100.1 ± 2.4 mg 100 ml^{-1} (overall mean; $P<0.001$; Fig. 4a). Plasma total protein levels were not significantly influenced by the feeding regime until between days 71 and 92. On days 92 and 120 plasma total protein concentration was significantly ($P<0.001$) lower in the fasted group (63.5, 58.3 mg 100 ml^{-1}) than the re-fed or control (82.4, 92.4 mg 100 ml^{-1}) groups.

[Insert Fig. 4]

3.6.4 Plasma amino acids

Plasma total amino acids increased significantly in all treatment groups between day 29 and day 120 (P<0.001: fed, re-fed; P < 0.05: fasted; Fig. 4b). However, on day 120, mean plasma total amino acid concentrations were significantly lower in the fasted fish (21.4 ± 0.7 mg 100 ml^{-1}) than in the fed (31.6 ± 3.1 mg 100 ml^{-1}) or re-fed groups (P<0.001).

3.6.5 Liver glycogen

Between days 29 and day 120 there was a small but significant (P<0.05) drop in liver glycogen concentration in the fed fish (Fig. 4c). Liver glycogen levels in both re-fed (116.5 mg g^{-1}) and fasted fish (117.9 mg g^{-1}) were significantly lower than those in the fed fish (211.6 mg g^{-1}) on day 29 (P<0.001). Following the resumption of feeding, levels in the re-fed group were statistically indistinguishable from those in the controls by day 71, while in the fasted fish levels remained significantly lower than those in the fed group, continuing to decline significantly (P < 0.05) until day 120 (179.0 c.f. 82.8 mg g^{-1}).

4. Discussion

This study was carried out using fish held in a semi-natural environment, subject to natural photoperiod and water temperature fluctuations. The 17 weeks duration of the study (mid-February to mid-June) spanned winter to late spring and re-feeding commenced at a point judged to correspond to the increase in availability of natural food. An all-female population of rainbow trout was employed to avoid the potential complications caused by the presence of early-maturing male fish and the study conditions therefore do not take into account any modulation of the response to fasting brought about by reproductive status. Blood borne indices of nutritional status were measured in order to provide information on the concentration of specific metabolites being

delivered to tissues at each sample point, at a temporal resolution comparable to that employed for the plasma hormone measurements.

4.1 Growth

Despite the continued availability of food to the control group there was no significant weight gain during the first half of the experimental period. Uneaten food was not collected and we don't therefore know whether the absence of growth was due to a reduced appetite or to direct effects of temperature on conversion efficiency. These observations are however consistent with studies of over-wintering salmon (*Salmo salar*) in which growth slows or ceases even in the presence of adequate rations because of alterations in appetite (Metcalfe and Thorpe, 1992; Simpson et al., 1996). The control fish in this study were clearly feeding at least to maintenance levels because they defended their body weight and condition, in contrast to the fasted fish. In continuously fed fish growth resumed as water temperatures exceeded 9^{o}C and during the subsequent 49 days mean body weight increased by 48%. Surprisingly, there are few data with which to directly compare these results. In a recent study on coho salmon (Larsen et al., 2001) fish held at 2.5^{o}C failed to gain weight whereas those held at 10^{o}C grew continuously. However, continued growth at 5^{o}C has been reported for rainbow trout (Brännäs and Wiklund, 1992). Both these studies employed artificial temperature regimes whereas fish in the present experiment experienced a temperature cycle that comprised part of the natural annual cycle, to which the fish had been exposed for several generations. Such differences in acclimation conditions are likely to contribute to variability between studies. A bioenergetics based growth model for rainbow trout indicated that growth would not occur in this species at 5^{o}C (Railsback and Rose, 1999). It is also possible that in addition to environmental temperature, photoperiod plays a part in dictating when growth resumes after winter (Boeuf and Falcon, 2001).

In the fasted treatment groups weight loss and a consequent decline in condition were evident. The major part of this weight loss (20% of starting weight) occurred during the first 29 days of fasting. Only a further 2% of initial body weight was lost during the remaining 91 days in the continuously fasted fish. This is broadly consistent with the loss of weight (32%) that occurred in rainbow trout starved for 91 days at a higher temperature (12^{o}C) than the present study (Weatherley and Gill, 1981) and in Atlantic salmon (11%) starved for 86 days at 3 - 6^{o}C (Einen et al., 1998). Re-feeding on day 63 resulted in an immediate gain in weight but re-fed fish failed to fully compensate for their period of fasting and displayed a lower mean weight (and shorter mean length, data not shown) than fully fed fish at termination of the study. However, condition factors of the continuously fed and the re-fed groups were indistinguishable at the end of the study suggesting that the re-fed fish were proportionately heavier for their length than the fed fish. There may therefore have been some compensatory hyperphagia among the re-fed fish (Bull and Metcalfe, 1997).

4.2 Metabolic indices

The response of fish to fasting is characterised by a sequential utilisation of glycogen, lipid and protein reserves (Collins and Anderson, 1995). In the present study, hepatic glycogen reserves were rapidly depleted in fasted fish and concomitant with this, plasma glucose levels declined significantly. This apparently counter-intuitive pattern may reflect the mobilisation of glycogen to replace the absence of dietary carbohydrate intake accompanied by a re-adjustment of the set-point for plasma glucose levels. Lower plasma glucose levels may have been tolerated because of the increased utilisation of lipid in fasted fish, reflected by the very marked elevation of plasma FFA levels within days of feed restriction starting. Elevated plasma FFA levels are a

characteristic marker of food withdrawal in fish (Farbridge and Leatherland, 1992). Re-feeding was accompanied by an extremely rapid decline in plasma FFA levels (evident within 24h of the recommencement of feeding) and a rapid elevation of blood glucose levels accompanying the replenishment of hepatic glycogen. Rapid recovery of plasma metabolites to pre-fasted levels has been reported for re-fed carp (Böhm et al., 1994). In continuously fasted fish the depression of plasma glucose levels was not consistent throughout the experimental period. After day 71, as water temperature rose above 9^{o}C, there was a pronounced elevation of plasma glucose levels accompanied by further depletion of hepatic reserves. This perhaps reflected additional metabolic demands brought about by the rising water temperature. During the final phase of the study, as both plasma glucose and plasma FFA levels declined again in the fasted fish, there was evidence of a switch to protein catabolism with both plasma total protein and plasma amino acids displaying a marked decline. The decline in total plasma amino acids was much delayed compared to changes reported for fasted brown trout, *Salmo trutta*, in which alterations were observed within days of food withdrawal (Navarro et al., 1997).

4.3 Cortisol

Plasma cortisol levels displayed some variation during the course of the experiment in all three treatment groups. However, no consistent treatment-related patterns could be discerned. Fasting appeared to have no direct effect on plasma cortisol levels. Overall, plasma cortisol levels were within the range expected of unstressed rainbow trout (Barton and Iwama, 1991) although some of the variability in the control group early in the experimental period suggests that there may have been some unaccounted-for source of disturbance. This may also have been the cause of the brief elevation of plasma glucose and SL in these fish during this period. These data therefore suggest that even under severe conditions of feed restriction cortisol does not appear to play a

functional role in mobilising energy in fish. However, caution must be exercised in extending this conclusion too broadly. Although this finding is consistent with some previous work in a range of species (Joergensen et al., 1999; Reddy et al., 1995; Holloway et al., 1994; Sumpter et al., 1991; Vijayan et al., 1993; White and Fletcher, 1986) it is in conflict with other reports that suggest that circulating cortisol levels are reduced (Barton et al., 1988; Farbridge and Leatherland, 1992a) or increased (Jorgensen et al., 2002; Blom et al., 2000; Kelley et al., 2001; Varnavsky et al., 1995) during fasting in fish. Furthermore, cortisol has clearly been shown to possess catabolic properties in teleost fish (Mommsen et al., 1999). Therefore, while the results of the present study provide no evidence that energy mobilisation in fasted rainbow trout is cortisol dependent, it cannot safely be assumed that this applies under all conditions.

4.4 GH

Plasma GH is widely reported to increase during periods of fasting in fish (Farbridge and Leatherland, 1992a,b; Holloway et al., 1994; Johnsson et al., 1996; Small et al., 2002; Sumpter et al., 1991; Takahashi et al., 1991; Varnavsky et al., 1995; Weber and Grau, 1999). This is suggested to occur because of a decline in hepatic GH receptors leading to reduced IGF-I feedback on somatotropes (Björnsson, 1997). In fasted coho salmon the decrease in GH receptors occurs within 3 weeks of the start of fasting (Gray et al., 1992) and GH levels are reported to rise within 3 weeks of food deprivation in fasted coho salmon (Varnavsky et al., 1995) and within 2 weeks of food deprivation in rainbow trout (Farbridge and Leatherland, 1992). The delayed response to fasting in salmonids compared to mammals has frequently been noted and may be a feature of heterothermic animals, such as fish, that are adapted to prolonged periods of fasting (Duan, 1998). In the present study we did not observe an unequivocal increase in plasma GH in fasted fish until 10 weeks had elapsed. In one group of fasted fish (subsequently re-fed) there was

a small but significant elevation in plasma GH relative to fed fish within four weeks of food withdrawal but this was not evident in the second group of fasted fish. The extent to which GH levels were elevated in the re-fed fish during the first 10 weeks was minimal (<4 ng ml^{-1}) and may have arisen due to an unaccounted for stressor although the reported effects of stress on GH levels are variable (Auperin et al., 1997; McCormick et al., 1998; Pickering et al., 1991) and there was no evidence of prolonged elevation of cortisol levels in these fish. The fish were all drawn from the same initial population and it therefore seems unlikely that past nutritional history contributed to this difference. Nonetheless, the changes in GH concentration, although minimal, were within the range reported by others in fasted fish (Holloway et al., 1994; Sumpter et al., 1991) so while inexplicable cannot be dismissed. In contrast, the alterations in plasma GH that occurred after 10 weeks in the fasted fish were considerably more pronounced than those that were evident during the earlier period and GH levels increased progressively in these fish for the remainder of the study. These data seem to counter the view that GH has a primary role in lipid mobilisation in fish. Previous studies have suggested that in fish GH stimulates lipid mobilization *in vitro* (O'Connor et al., 1993) and inhibits lipogenic enzymes (Leena et al., 1999), effects similar to those attributed to GH in mammals (Richelsen, 1997). The results of the present study depict a rapid and sustained elevation of plasma FFA following food withdrawal that is not linked to elevated GH. Even in the refed group that displayed a modest elevation of GH, elevation of FFA preceded changes in plasma GH by several weeks.

The increase in GH in fasted fish after 10 weeks was closely related to water temperature which rose from approximately 8°C to 16°C during the same period. Given that there was no change in GH levels during this period in fed fish it is unlikely that this represents a simple relationship between temperature or photoperiod and GH (Björnsson, 1997; Björnsson et al., 2000; Pérez-

Sanchéz and Le Bail, 1999). It is more likely that the changing thermal environment exacerbated the deficit in available energy in fasted fish by driving the metabolic rate of the fish upwards and this was responsible for the nine-fold increase in GH levels. If the view is taken that high plasma GH levels can consistently be linked to an increased catabolism:anabolism ratio in fish (Pérez-Sanchéz and Le Bail, 1999) these data appear to indicate that overwintering rainbow trout are not energetically compromised by food withdrawal if energy reserves are adequate to sustain the level of metabolic activity that is required. It may only be when a significant imbalance occurs that GH levels rise, exemplified by the conditions during the phase of this study when water temperature rose above 9ºC. In the fasted fish during this stage the elevated GH levels coincided with declining plasma FFA levels, increasing depletion of hepatic glycogen and the presumptive onset of protein utilisation. Administration of bovine GH to tilapia (Leung *et al*., 1991) resulted in a significant decline in hepatic glycogen levels and a reduction in activity of glycogen synthetase, and, in smolting salmonids a decline in hepatic glycogenesis and increase in glycogenolysis (Sheridan *et al*., 1985) coincide with increasing levels of GH (Young *et al*., 1989). GH apparently has little effect on protein degradation in fish (Foster *et al*., 1991) so the causal factor underlying the utilisation of protein and AA (assuming that this is what a decline in plasma concentrations indicates) is not evident from these data.

There are no studies, in which fasted fish were monitored over a prolonged period against a natural temperature regime, with which to directly compare these results. In rainbow trout that were subject to a temperature drop from 10ºC to 2.5ºC and simultaneously fasted there was a decline in IGF-1 levels (Larsen et al., 2001) that might therefore have been expected to cause an elevation in GH levels (although GH was not measured). However, IGF-1 levels also declined in the fed fish exposed to the same temperature drop. We found no evidence for changes in GH

levels in the fed fish throughout the present study.

4.5 Somatolactin

Significant treatment-related differences in plasma SL levels were evident at two stages of the study. Elevation of SL in the control, fed, fish during the first week of the study were coincident with similar alterations in both cortisol and glucose, strongly suggesting that these fish experienced a minor stressful disturbance. Plasma SL levels are known to rise in trout exposed to stressors (Rand-Weaver et al., 1993). As was the case for GH, alterations in SL levels during fasting were not consistently related to treatment. SL levels were significantly depressed in only one group of fasted fish, and this was not the group in which minor changes in GH were observed. However, after the resumption of feeding, SL levels in the continuously fasted group remained significantly lower than in both fed groups. That SL levels remain low during prolonged fasting in trout, while GH levels rise, has been commented on previously (Rand-Weaver et al., 1995) and is consistent with the relative activities of pituitary GH and SL cells in fasted eels (*Anguilla anguilla*; Olivereau and Olivereau, 1997) which are inversely correlated. However, in this study the magnitude of change in SL levels was much more limited than that observed for GH and rather than a decline in response to fasting, might be described as a failure of SL levels in fasted fish to rise with increasing temperature. It has been shown in a previous study that plasma SL levels in trout are positively correlated with water temperature (Rand-Weaver et al., 1995). The inconsistency of the SL response to fasting, contrasted with the uniformity of the changes in FFA and glucose following food withdrawal, and the lack of change in SL during prolonged fasting suggest that in trout this hormone exerts no active effect on energy mobilisation and plays a passive role during fasting. This conclusion is at odds to that reached by Company et al. (2001) who observed a transient elevation of SL in response to fasting

in gilthead sea bream (*Sparus aurata*). A subsequent detailed study of seasonal variation in SL in sea bream (Mingarro et al., 2002) reports that in this species plasma SL levels are at their lowest when water temperatures are warmest and begin to rise as water temperatures decline. These authors consequently proposed that SL plays an active role in the response to fasting. To reconcile these apparently contradictory conclusions requires further study.

5. Conclusion

Several conclusions are evident from the results of this study, the extent to which these can be applied more widely is at present unclear: (i) the effects of food withdrawal on indices of carbohydrate and lipid mobilisation in rainbow trout were rapid but were quickly reversed when feeding resumed; (ii) indicators of protein catabolism in rainbow trout were affected only after a very prolonged period of fasting; (iii) sustained mobilisation of lipid reserves in rainbow trout occurred in the absence of elevated plasma GH levels; (iv) plasma GH levels in rainbow trout did not increase significantly and consistently until after several weeks of fasting; (v) plasma GH levels did not rise in fed trout with increasing spring water temperatures or lengthening photoperiod; (vi) plasma cortisol levels in rainbow trout were not affected by fasting; (vii) there was no evidence for direct involvement of SL in the response to fasting. Some of these observations are consistent with what is already known about the metabolic and endocrine impact of fasting in fish, some are not. In particular, the fact that overwinter fasting was not accompanied by alterations in GH suggests that elevation of GH is not an inevitable consequence of food withdrawal. The failure of plasma cortisol levels to exhibit a consistent response to food withdrawal suggests that cortisol may not necessarily play a role in energy mobilisation under extreme nutritional restriction. This finding also suggests that fasting *per se* is not perceived by the fish as a stressor, given that there was no evidence for activation of the hypothalamic-

pituitary-interrenal axis. To fully understand the endocrine processes underlying the response to fasting in fish may require a multi-factorial study of a duration and complexity not previously attempted, and/or the harnessing of post-genomic technologies.

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Captions to figures

Figure 1. Changes with time in (a) water temperature (°C); (b) body weight; (c) coefficient of condition ((100 x W) / l^3) in control, fed, rainbow trout (○), fasted rainbow trout (△) and in fasted, re-fed, rainbow trout (●). The period during which food was withdrawn is denoted by the grey bar. Each point is the mean ± SEM, n = 20. Asterisks indicate significant differences from control, fed fish; * P<0.05, ** P<0.01, *** P<0.001.

Figure 2. Changes with time in (a) plasma cortisol; (b) plasma GH; (c) plasma SL in control, fed, rainbow trout (○), fasted rainbow trout (△) and in fasted, re-fed, rainbow trout (●).The period during which food was withdrawn is denoted by the grey bar. Each point is the mean ± SEM, n = 8 (cortisol); 20 (SL, GH). Asterisks indicate significant differences from control, fed, fish; * P<0.05, ** P<0.01, *** P<0.001.

Figure 3. Changes with time in (a) plasma FFA; (b) plasma glucose, in control, fed, rainbow trout (○), fasted rainbow trout (△) and in fasted, re-fed, rainbow trout (●).The period during which food was withdrawn is denoted by the grey bar. Each point is the mean ± SEM, n = 20. Asterisks indicate significant differences from control, fed, fish; * P<0.05, ** P<0.01, *** P<0.001.

Figure 4. . Changes with time in (a) plasma total protein; (b) plasma total amino acids; (c) liver glycogen in control, fed, rainbow trout (○), fasted rainbow trout (△) and in fasted, re-fed, rainbow trout (●).The period during which food was withdrawn is denoted by the grey bar. Each point is the mean ± SEM, n = 10 (AA, glycogen); 20 (protein). Asterisks indicate significant differences from control, fed, fish; * P<0.05, ** P<0.01, *** P<0.001.

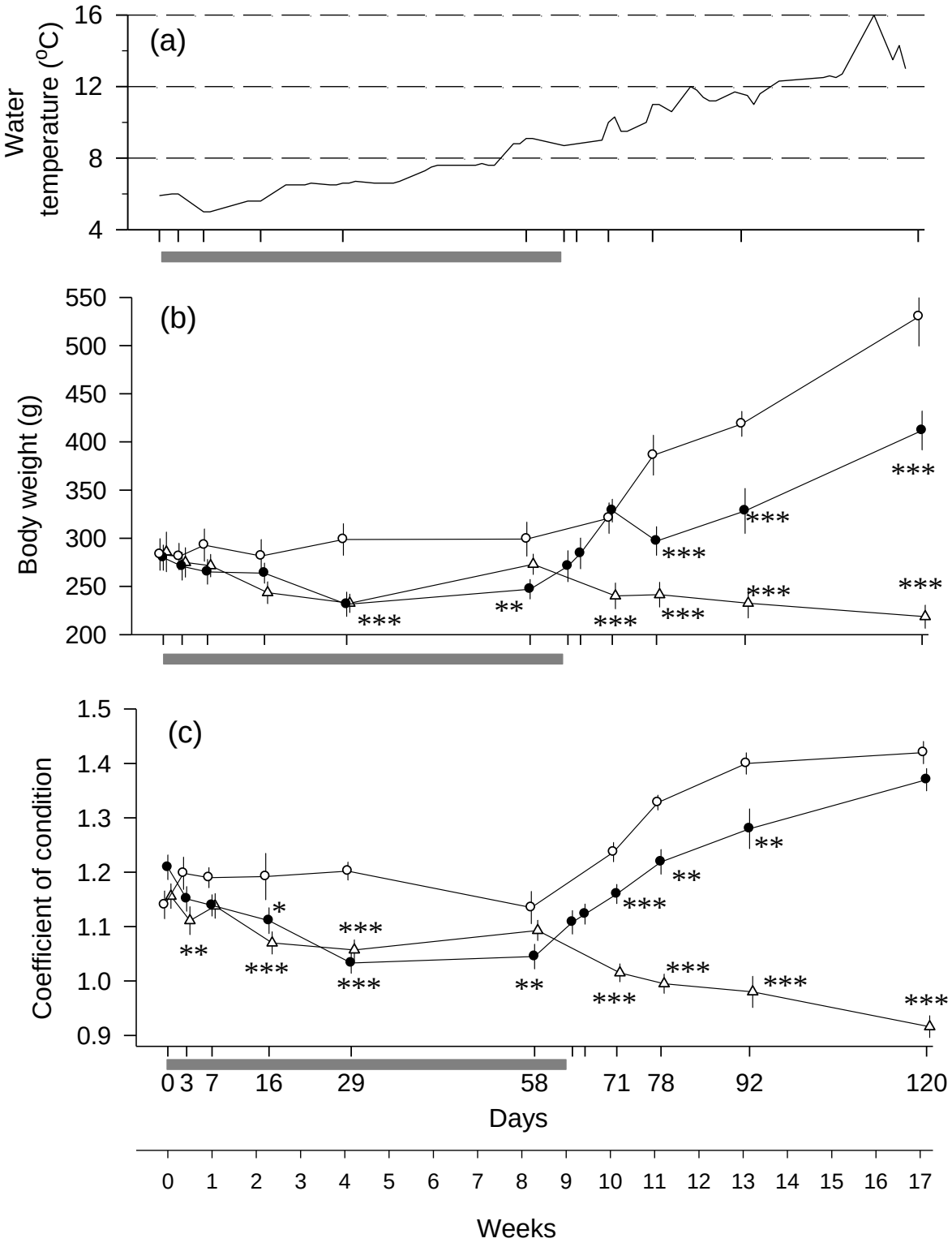
(a)
Water temperature (°C)
16
12
8
4
(b)
Body weight (g)
550
500
450
400
350
300
250
200
(c)
Coefficient of condition
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0 3 7 16 29 58 71 78 92 120
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Weeks

Figure 1.

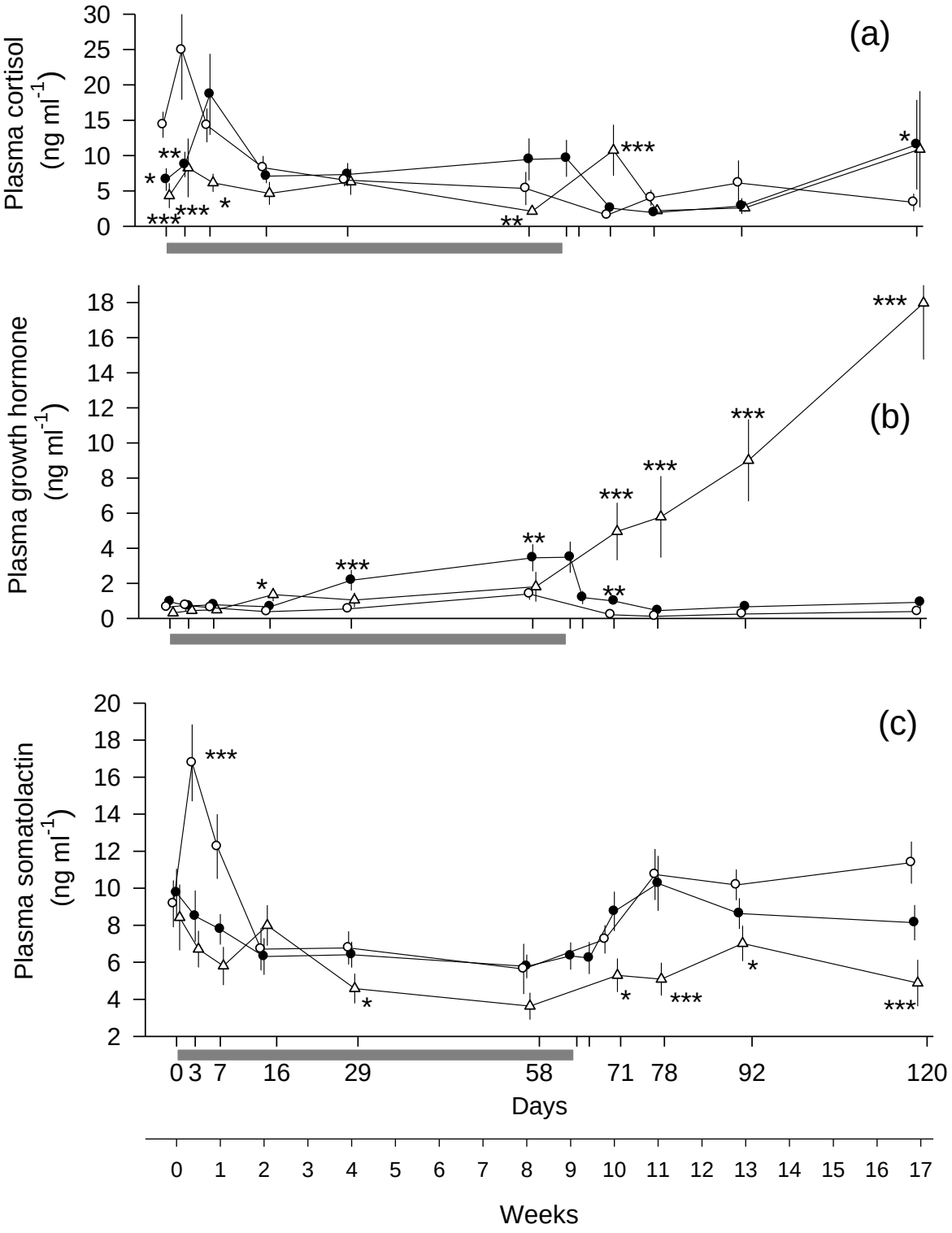
(a)
Plasma cortisol (ng ml-1)
30
25
20
15
10
5
0
(b)
Plasma growth hormone (ng ml-1)
18
16
14
12
10
8
6
4
2
0
(c)
Plasma somatolactin (ng ml-1)
20
18
16
14
12
10
8
6
4
2
0 3 7 16 29 58 71 78 92 120
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Weeks

Figure 2.

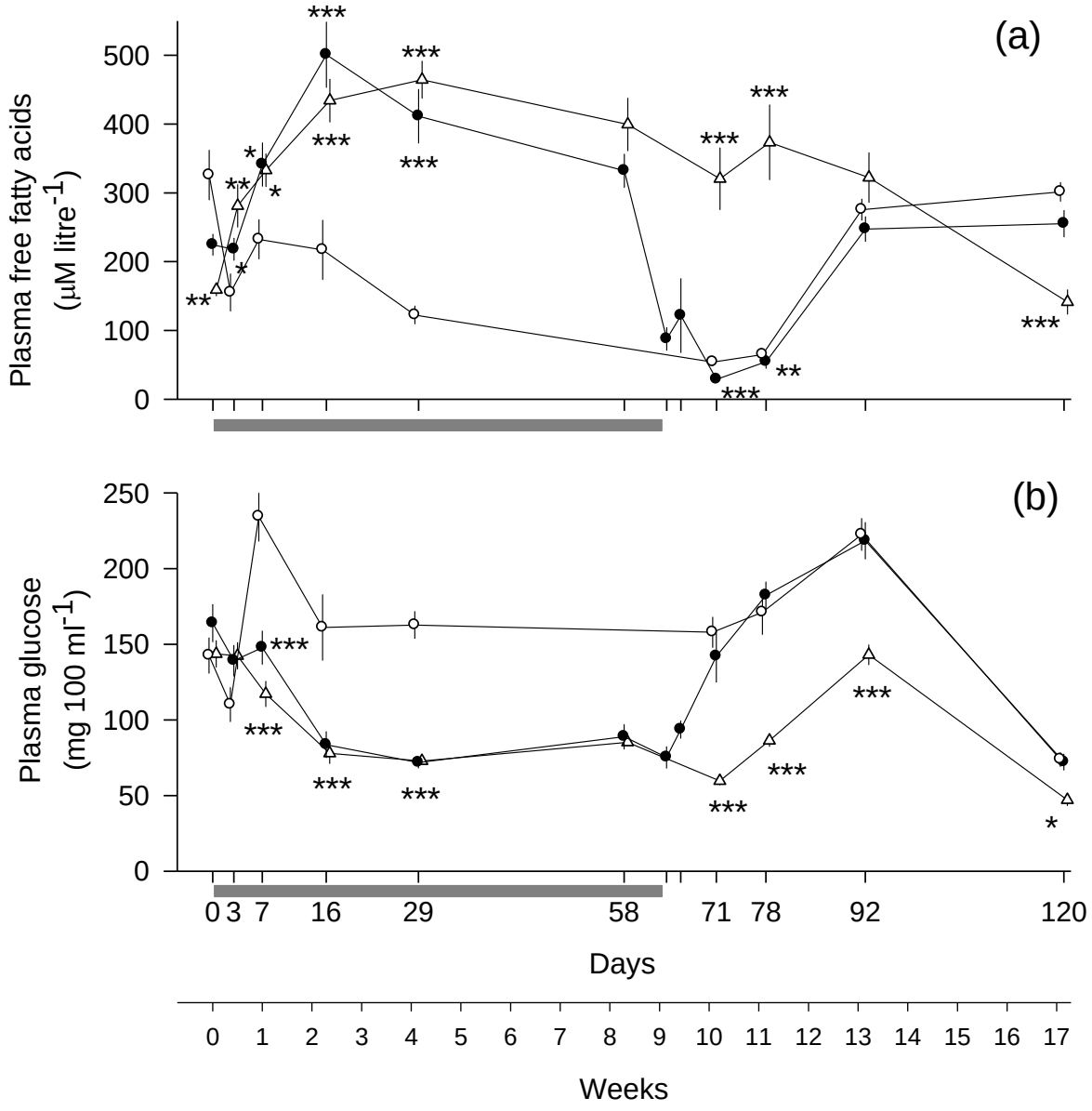
(a)
Plasma free fatty acids
(μM litre-1)
500
400
300
200
100
0
(b)
Plasma glucose
(mg 100 ml-1)
250
200
150
100
50
0
0 3 7 16 29 58 71 78 92 120
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Weeks

Figure 3.

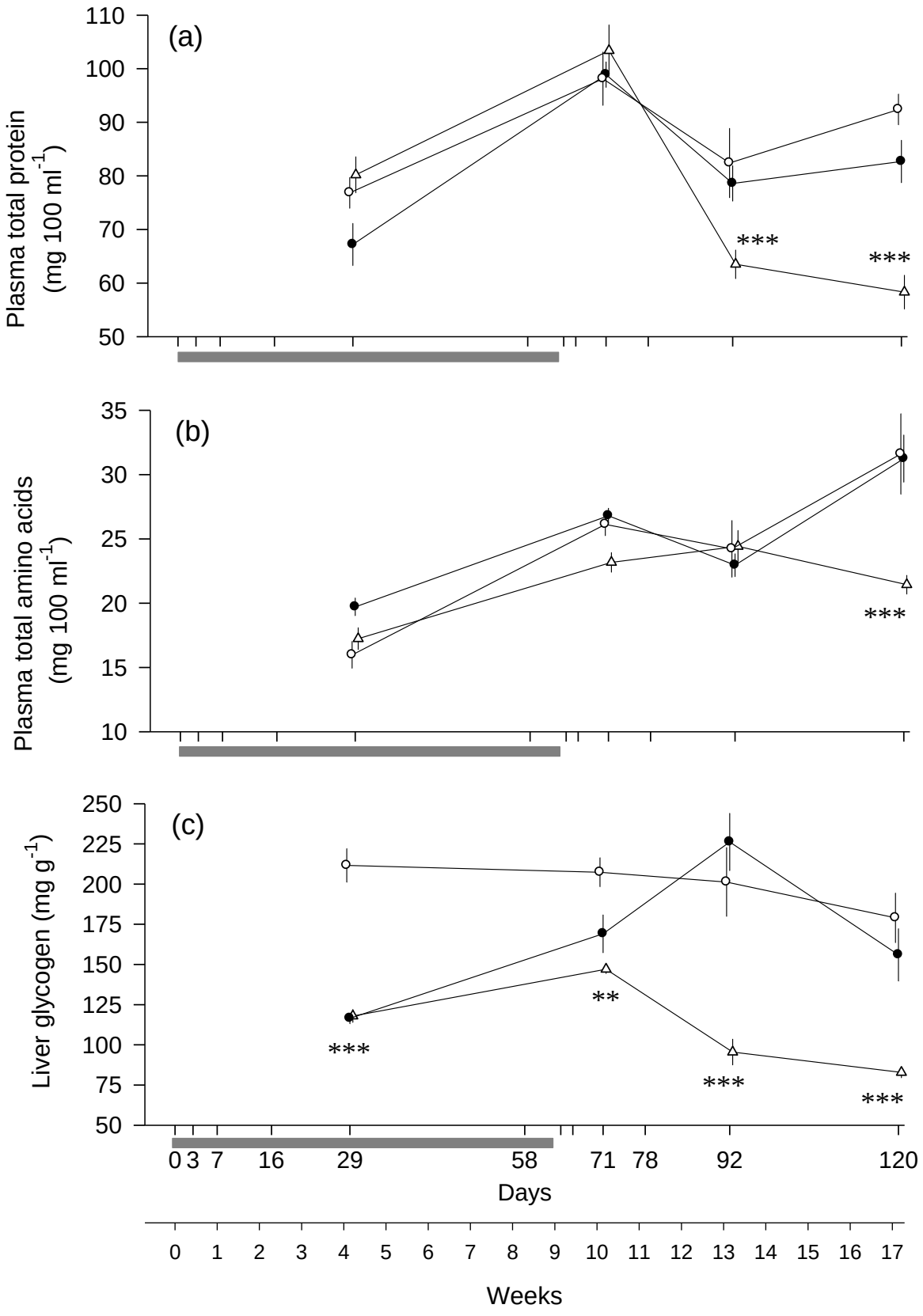
(a)
Plasma total protein (mg 100 ml-1)
110
100
90
80
70
60
50
***
***
(b)
Plasma total amino acids (mg 100 ml-1)
35
30
25
20
15
10
***
(c)
Liver glycogen (mg g-1)
250
225
200
175
150
125
100
75
50
***
**
***
***
0 3 7 16 29 58 71 78 92 120
Days
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Weeks

Figure 4.