Chronic cortisol and the regulation of food intake and the endocrine growth axis in rainbow trout

Barry N Madison, Sara Tavakoli, Sarah Kramer and Nicholas J Bernier
Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada N1G 2W1

Abstract

To gain a better understanding of the mechanisms by which cortisol suppresses growth during chronic stress in fish, we characterized the effects of chronic cortisol on food intake, mass gain, the expression of appetite-regulating factors, and the activity of the GH/IGF axis. Fish given osmotic pumps that maintained plasma cortisol levels at ~70 or 116 ng/ml for 34 days were sampled 14, 28 and 42 days post-implantation. Relative to shams, the cortisol treatments reduced food intake by 40–60% and elicited marked increases in liver leptin (lep-a1) and brain preoptic area (POA) corticotropin-releasing factor (crf) mRNA levels. The cortisol treatments also elicited 40–80% reductions in mass gain associated with increases in pituitary gh, liver gh receptor (ghr), liver igfl and igf binding protein (igfbp)-1 and -2 mRNA levels, reduced plasma GH and no change in plasma IGF1. During recovery, while plasma GH and pituitary gh, liver ghr and igfl gene expression did not differ between treatments, the high cortisol-treated fish had lower plasma IGF1 and elevated liver igfbp1 mRNA levels. Finally, the cortisol-treated fish had higher plasma glucose levels, reduced liver glycogen and lipid reserves, and muscle lipid content. Thus, our findings suggest that the growth-suppressing effects of chronic cortisol in rainbow trout result from reduced food intake mediated at least in part by increases in liver lep-a1 and POA crf mRNA, from sustained increases in hepatic igfbp1 expression that reduce the growth-promoting actions of the GH/IGF axis, and from a mobilization of energy reserves.

Key Words
- chronic stress
- food intake
- growth regulation
- GH/IGF1 system
- fish

Introduction

The chronic stress associated with a variety of adverse environmental conditions has been shown to suppress somatic growth in fish. For example, prolonged crowding (Pickering & Pottinger 1989, Trenzado et al. 2006), repeated handling (Barton et al. 1987, Hoskonen & Pirhonen 2006), poor water quality (Schram et al. 2009) and social subordination (DiBattista et al. 2006) are common chronic stressors in aquaculture that reduce growth rates. These stressors also activate the hypothalamic–pituitary–interrenal axis and as a result are associated with an increase in the circulating levels of the primary glucocorticoid in teleosts fish, cortisol (Wendelaar Bonga 1997, Bernier et al. 2009). Since glucocorticoids promote the mobilization and redistribution of energy, the effects of chronic stress on growth in fish have generally been attributed to the actions of cortisol (Pickering 1992, Pankhurst & Van Der Kraak 1997, Small et al. 2008). Although studies using exogenous
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Cortisol have previously linked the growth-suppressing effects of cortisol in fish to its actions on food intake, intermediary metabolism and muscle mass regulation (reviewed by Mommsen et al. (1999), Bernier (2006) and Picha et al. (2008)), the precise mechanisms by which cortisol affects these complex systems are far from clear.

Cortisol affects food intake in fish but its primary effect and mechanism of action are unresolved. While the primary effect of glucocorticoids in mammals and amphibians is to increase food intake (Crespi et al. 2004, Bazhan & Zelena 2013), the actions of cortisol on appetite in fish are equivocal. Whereas chronic exposure to a low dose of cortisol stimulated food intake in goldfish (Carassius auratus; Bernier et al. 2004), sustained exposure to higher doses of cortisol decreased food intake in several fish species (Gregory & Wood 1999, Bernier et al. 2004, Peterson & Small 2005, Leal et al. 2011). In mammals, glucocorticoids promote food intake by stimulating the hypothalamic expression of the orexigenic neuropeptides neuropeptide Y (NPY) and agouti-related peptide (AgRP), and by inhibiting the anorexigenic signal corticotropin-releasing factor (CRF; Bazhan & Zelena 2013). Similarly, the stimulatory effects of cortisol on food intake in goldfish are associated with an increase in hypothalamic NPY mRNA levels and a reduction in preoptic area (POA) CRF gene expression (Bernier et al. 2004). In contrast, the pathways mediating the anorexigenic effects of cortisol in fish have yet to be identified (Bernier et al. 2004, Leal et al. 2011). In mammals, glucocorticoids are a potent stimulator of adipocyte leptin expression and secretion, and under conditions of glucocorticoid excess leptin can counteract the orexigenic effects of cortisol (Leal-Cerro et al. 2001, Lee & Fried 2009). The primary site of leptin expression in fish is the liver (Londraville et al. 2014). Whether the anorexigenic effects of cortisol in fish are at least partly mediated by an increase in hepatic leptin expression is not known.

Somatic growth in fish, as in other vertebrates, is primarily mediated by the growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis (reviewed by Wood et al. (2005) and Reindl & Sheridan (2012)) and to better understand how stressors impact growth several studies have recently described the effects of exogenous cortisol and stressors on the activity of this endocrine system. However, despite the fact that growth suppression is a feature of chronic stress, the large majority of these studies only focused on the short-term effects of stress (i.e. <48 h). Overall, on an acute time scale, cortisol injections or stressors in fish either have no effect or decrease plasma GH levels, reduce liver igf1 gene expression and plasma IGF1 levels, counteract GH stimulation of liver igf1 transcription, and increase the hepatic expression and plasma levels of low molecular weight IGF binding proteins (IGFBPs) (Kelley et al. 2001, Kajimura et al. 2003, Pierce et al. 2005, Wilkinson et al. 2006, Leung et al. 2008, Saera-Vila et al. 2009, Breves et al. 2010, Shepherd et al. 2011, Nakano et al. 2013). In channel catfish (Ictalurus punctatus), chronic dietary cortisol had no effect on liver igf1 mRNA levels, reduced plasma IGF1, and increased the plasma levels of low molecular weight IGFBPs (Peterson & Small 2005). Whether chronic cortisol exposure has similar effects on the GH/IGF1 system in other fish species or whether these effects are sustained once cortisol has returned to baseline levels have not been investigated.

In this study, to identify the mechanisms by which chronic cortisol suppresses growth in rainbow trout (Oncorhynchus mykiss), we characterized the effects of a 34-day period of sustained moderate physiological levels of cortisol on food intake, mass gain, the plasma levels of GH and IGF1, and the gene expression of key appetite- and growth-regulating factors. To determine whether the cortisol dosages used promote the mobilization of energy, we assessed the status of liver and white muscle carbohydrate and lipid reserves. To gain a better understanding of whether the effects of chronic cortisol are sustained once cortisol levels have returned to baseline, we also quantified the above parameters 8 days post cortisol exposure. Finally, we used trout hepatocytes in primary culture to determine whether the in vivo stimulatory effects of cortisol on liver leptin gene expression are direct.

Materials and methods

Experimental animals

Sexually immature rainbow trout (n=72; 192±3 g) of mixed sex were obtained from Rainbow Springs Trout Farm (Thamesford, ON, Canada) and housed at the University of Guelph. Fish were acclimated in 700-l fiberglass tanks with flow-through well water on a 12 h light:12 h darkness photoperiod for several weeks prior to the onset of experiments. Water conditions were maintained at 12±1 °C, pH of 7.8±0.2 during the acclimation and the experimental period. Fish were fed 2% body weight (BW) daily with commercial trout food (3 pt Classic Sinking, Martin Mills, Elmira, ON, Canada). All procedures were carried out in accordance with the Canadian Council for Animal Care guidelines and approved by the University of Guelph Animal Care Committee (protocol 05R074).

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Experimental protocol

In vivo cortisol treatment  Fish were anesthetized in buffered (NaHCO₃, 0.2 g/l) tricaine methanesulfonate (0.1 g/l, Syndel International, Qualicum Beach, BC, Canada), weighed and implanted intramuscularly with a 125 KHz passive integrative transponder (11.5×2.1 mm, Biomark, Boise, ID, USA) into the peritoneal cavity. While control fish (Sham) (Alzet model #1007D, Durect Corporation, Cupertino, CA, USA) were implanted with pumps containing a 65% solution of the steroid miscible vehicle 2-hydroxypropyl-ß-cyclodextrin (Molecusol HBP, Sigma–Aldrich), cortisol groups were implanted with pumps containing 20 µg/g BW (Cort-I) or 40 µg/g BW (Cort-II) cortisol (hydrocortisone, Sigma–Aldrich) dissolved in 65% Molecusol HBP. Molecusol HBP was used as a non-toxic complexing agent to increase the aqueous solubility of cortisol and minimize the size of the micro-osmotic pumps needed to perform the chronic exposure. The cortisol dosage levels were chosen on the basis of plasma cortisol concentrations achieved during a pilot study. At a water temperature of 12°C, it was calculated and confirmed in preliminary experiments that the osmotic pumps deliver cortisol for a period of 34 days. Sham, Cort-I and Cort-II fish were terminally sampled at 14, 28 and 42 days post osmotic pump implantation.

At the time of terminal sampling, all fish in a given tank were anesthetized at once with a lethal dose of 2-phenoxyethanol (2 ml/l, Sigma–Aldrich) and a blood sample was immediately taken via caudal puncture using a K₂EDTA (0.5 M, pH 8.0)-treated syringe. Blood samples were immediately centrifuged at 14,000 g for 3 min and the separated plasma was aliquoted and flash frozen in liquid nitrogen prior to storage at −80°C for later analysis of plasma cortisol, adrenocorticotropic hormone (ACTH), GH, IGF1 and glucose. Fork length and BW were then recorded, and several tissues were collected to quantify the mRNA levels of key appetite- and growth-regulating genes by quantitative real-time RT-PCR (qRT-PCR) or tissue metabolite levels. The brain was removed and regionally dissected (Bernier et al. 2008) to isolate the POA and the hypothalamus. Whole pituitary and liver, and a standardized sample of white muscle (rostral to dorsal fin) were also collected. All tissues were immediately frozen in liquid nitrogen and stored at −80°C for future analysis.

Primary culture of trout hepatocytes  To determine whether the actions of cortisol on hepatic leptin-A1 gene expression (lep-1a) are direct and mediated by glucocorticoid receptors (GR), we exposed primary cultures of trout hepatocytes in vitro to cortisol or a combination of cortisol and RU486 (a GR antagonist). Trout hepatocytes were isolated by in situ perfusion of liver with collagenase (Sigma) as previously described (Sathiyaa et al. 2001). Trypan blue dye exclusion method was used to confirm hepatocyte viability and >95% cells were viable. Cells were plated in six-well tissue culture plates (Sarstedt, Inc., Newton, NC, USA) at a density of 1.5 million cells/well (0.75 million cells/ml) in L-15 media and were maintained at 12°C for 24 h. After 24 h, the L-15 media was replaced with either fresh L-15 (control; containing 0.01% ethanol as vehicle), L-15 containing cortisol (100 ng/ml), or L-15 with a combination of cortisol (100 ng/ml) and RU486 (1000 ng/ml), and cells were maintained at 12°C for 24 h before sampling. In the combination treatment, RU486 was added to the cells 30 min before cortisol addition. All the experiments were repeated with hepatocytes isolated from six different fish.

Analytical techniques

Food intake quantification  To prevent the formation of within tank social hierarchies, fish were carefully selected to minimize size differences within and between treatments, and fish in each tank were slowly hand fed to satiation daily throughout the acclimation and experimental periods. Individual food intake was quantified by X-ray radiography on the day of terminal sampling (Bernier & Craig 2005). Fish were fed to satiation with re-pelleted food containing 450 µm carbon steel beads (Draiswerke, Inc., Mahwah, NJ, USA) at a ratio of 5% by mass of dry powdered feed. Ninety minutes following feeding fish were terminated with an overdose of 2-phenoxyethanol as above and X-rayed using an ACU-RAY HFJ portable X-ray unit (50 kVp, 1.05 mAs @ 90 cm; Sterne, ON, Canada). After development of the radiographs, the number of steel beads in the gastrointestinal tract was tallied and the amount of feed consumed calculated using a calibration curve.

Quantification of growth rate and physical indices  Specific growth rate (SGR, in % BW/d) was derived from initial BW (W₀), final BW (W₁) and the length of the sampling interval (t, in days), and calculated as: SGR = ((ln W₁ − ln W₀) / t) × 100.
Plasma analyses  Plasma cortisol concentrations were measured in triplicate by RIA as per the methods of Bernier et al. (2008). The lower detection of the assay was 16 pg/ml. The intra- and inter-assay coefficients of variation were 3.2% (n = 6) and 5.3% (n = 6) respectively. Plasma ACTH levels were measured in duplicate following the procedures outlined in the ImmucChem Double Antibody hACTH RIA kit (product code #07-106101; MP Biomedicals, Orangeburg, NY, USA). This assay was previously validated for use with rainbow trout plasma (Craig et al. 2005). All samples were analyzed in one assay that had a lower detection limit of 10 pg/ml and an intra-assay variation of 5.1% (n = 8). Furthermore, a serial dilution of rainbow trout plasma gave a displacement curve parallel to the ACTH standard curve. Plasma IGF1 levels were assayed as described in Shimizu et al. (2000). Briefly, total IGF1 was first separated from binding proteins within the plasma by acid–ethanol and quantified using recombinant trout IGF1 as standard and tracer, and anti-recombinant barramundi IGF1 as primary antibody (1:7000; Novozymes GroPep Ltd, Adelaide, Australia). Plasma GH levels were assayed as described by Swanson (1994) using recombinant trout GH as standard and tracer and anti-recombinant trout GH as primary antibody (1:25 000; Novozymes GroPep Ltd). IGF1 and GH were iodinated by the chloramine-T method. For both the IGF1 and GH RIAs, 7000 cpm of tracer in 50 μl was added to tubes containing 50 μl of sample, 50 μl of primary antibody and 150 μl of RIA buffer (30 mM NaH2PO4, 0.02% protamine sulphate, 10 mM EDTA, 0.025% NaNO3, 0.05% (v/v) Tween-20, pH 7.5). After a 48 h incubation at 4 °C, the antibody-bound IGF1 or GH were complexed with secondary antibody (1:15, goat anti-rabbit IgG; AbD Serotec, Oxford, UK) and polyclonal rabbit anti-human IgG (1:200; Dako, Glostrup, Denmark), and precipitated by using ice-cold polyethylene glycol 6000 (PEG-6000; Sigma–Aldrich) and centrifugation at 4000 g for 30 min. Plasma IGF1 and GH analyses were each performed in a single assay. The lower detection limit of each RIA was <0.5 ng/ml and the intra-assay coefficients of variability for the IGF1 and GH assays were 4.0% (n = 6) and 0.5% (n = 6) respectively. Concentrations of plasma cortisol, ACTH, IGF1 and GH were determined using three-parameter sigmoideal curve regression equations (SigmaPlot 10, SPSS) obtained from the standard curves. Plasma glucose was determined using the standard NAD+-coupled enzymatic procedure described in Bergmeyer (1985) and modified for use with microplates and a SpectraMAX 190 spectrophotometer (Molecular Devices, Menlo Park, CA, USA).

Tissue metabolite analyses  Tissue glycogen content was measured using a modification of the method described by Bergmeyer (1985). Approximately 100 mg of liver or white muscle was homogenized (Euro Turrax T20b, IKA Labortechnik, Staufen, Germany) on ice for 1 min at approximately 12000 g in acetate buffer, pH 4.8 (Bergmeyer 1985). Glycogen in the resultant homogenate was converted to glucose by amylloglucosidase and free glucose was measured using an NAD+-coupled enzymatic reaction catalyzed by hexokinase and modified for use with a microplate spectrophotometer. Total liver and white muscle lipid content was gravimetrically measured on a separate set of tissue samples (~100 mg) using the 2:1 chloroform-methanol extraction method of Folch et al. (1957). Water content of liver and muscle tissue was determined by weight differential via desiccation in an oven at 70 °C for 2 days. All metabolite measures were corrected for tissue water content.

Quantification of gene expression  Tissues were homogenized and total RNA extracted using Trizol Reagent (Life Technologies). RNA pellets were then re-dissolved in RNase-free water and quantified by ultraviolet spectrophotometry at 260 nm (Nanodrop 8000; Nanodrop Products, Wilmington, DE, USA). Integrity of the isolated RNA was ensured prior to cDNA synthesis by analyzing a random subset of samples on a 1.5% agarose gel. One microgram of total RNA was treated with DNase I (Life Technologies) and reverse transcribed to cDNA using Superscript II RNase H-reverse transcriptase (Life Technologies) according to the manufacturer’s protocol. Non-reverse transcribed (no-RT controls) representatives from each tissue and treatment were included during cDNA synthesis to monitor genomic contamination. Triplicates of each cDNA sample were amplified by real-time PCR using an ABI Prism 7000 sequence detection system (Applied Biosystems). The 20 μl reactions contained 10 μl 2X SYBR Green PCR Master Mix (Applied Biosystems), 5 μl of 15-fold diluted first strand cDNA template or no-RT
controls and 2.5 μl of both forward and reverse primers (0.4 μM). Default cycling conditions were used and followed by a melting curve analysis to verify the specificity of each PCR product. Only samples with a unimodal dissociation curve and predicted melting temperature were analyzed. Primer pairs for qPCR were designed using Primer Express 3.0 (Applied Biosystems) based on rainbow trout sequences for each targeted gene (Table 1). To account for differences in amplification efficiency, standard curves were constructed for each gene using known dilutions of cDNA from the targeted tissues. Input values for each gene were obtained by fitting the average threshold cycle (C_T) value to the antilog of the gene-specific standard curve thereby correcting for differences in primer amplification efficiency. To correct for minor variations in template input and transcriptional efficiency, the input values were normalized to the housekeeping gene elongation factor 1α (ef1α). Note that the expression of ef1α did not differ between any of the treatments (P>0.05). Gene expression data is reported as fold change relative to the 14-day Sham treatment mean value.

### Statistical analyses

Results are presented as mean ± S.E.M. A two-way ANOVA followed by a Holm-Sidak post-hoc test for multiple comparisons was used to determine the effects of treatment and time on all the parameters measured in the in vivo cortisol treatment. A one-way ANOVA followed by a Holm-Sidak test was used to determine the effects of treatment on lep-a1 mRNA levels in the in vitro cultured hepatocytes. Data that did not meet the assumption of normality were log-transformed prior to analysis. Data presented in percentage were arcsine transformed prior to analysis. The relationships between plasma IGF1 levels and SGR within the Sham, Cort-I and Cort-II treatments were analyzed using Pearson’s Product Moment Correlation test. All analyses were performed using SigmaPlot.

### Table 1  Nucleotide sequences of rainbow trout primers used for qRT-PCR

| Gene | Accession no. | Efficiency (%) | Sequence (5’–3’)
|------|--------------|----------------|-----------------
| agrp | CR376289 | 90.8 | F: ACCACAGCTCTGTCTGGGTAA
| cart | CA380644 | 91.7 | R: AGTAGAGATGGCGCAAGAA
| crf | AF296672 | 90.8 | F: CCTCGACACAAGAAGTGTGAGAGA
| ef1α | AF498320 | 96.0 | R: TGTAGTGCTCCAAGCAGTTGCT
| gh1 | M22731 | 101.5 | F: ACAACGACTCAACTGAAGATCTCG
| gh2 | M24684 | 94.3 | R: AGGAAATTGAGCTTCATGTCAGG
| ghr2α | M6918 | 92.1 | F: CCATTGACATTTCGTTGAAAT
| igf1 | M95183 | 92.1 | R: GAGGTACCAGTGATCATGTTCTA
| igfbp1 | NM_001124561 | 91.0 | F: TCAAGAAGGAGCATGCAAAGGT
| igfbp2 | DQ146968 | 91.1 | R: TCTTCACGCACCACGTCAAGA
| lep-a1 | AB354909 | 95.1 | F: GCCGGAGTCGCTGGACTATGG
| npy | AF203902 | 99.8 | R: ATGGATCAGTGGTAGCTGCAG
| pomc-a2 | X69808 | 87.4 | F: TGGAGACACATCGTGGAACCA
| | | | R: CATTCCAGGAGTCCACATG

**agrp, agouti-related protein; cart, cocaine- and amphetamine-regulated transcript; crf, corticotropin-releasing factor; ef1α, elongation factor 1α; f, forward; gh, growth hormone; gh2, GH receptor type 2; igf1, insulin-like growth factor I; igfbp, IGF binding protein; lep-a1, leptin-A1; npy, neuropeptide Y; pomc-a2, pro-opiomelanocortin A2; r, reverse.**

*Primers for ghr2 amplify coding regions that are 100% homologous between the GHR2a and GHR2b paralogues (see Reindl & Sheridan (2012) for nomenclature).
12.5 (SPSS). The significance level for all statistical tests was \( P < 0.05 \).

**Results**

**Plasma cortisol, plasma ACTH and pituitary pomc gene expression**

Plasma cortisol in the three Sham groups did not differ from one another and averaged 6.0 ± 0.5 ng/ml. Fish implanted with cortisol-filled osmotic pumps had consistent plasma cortisol concentrations of 69.5 ± 0.9 ng/ml (Cort-I) and 115.7 ± 1.7 ng/ml (Cort-II) over the 14 and 28-day sampling periods (Fig. 1A). At 42 days, 8 days after the pumps had run out, the cortisol-treated fish had plasma cortisol concentrations that did not differ from the Sham treatment. Plasma ACTH concentrations in the cortisol-treated fish were reduced to less than half of the levels observed in the Sham treatment at 14 and 28 days (Fig. 1B). At 42 days, while plasma ACTH levels in the Cort-I treatment returned to the basal value of the Sham treatment, they remained low in the Cort-II treatment. Pituitary pro-opiomelanocortin A2 (pomc-a2) mRNA levels in the Cort-I and -II treatments were lower than in the Sham treatment during the first 14 and 28 days of the experiment, respectively, and recovered to basal levels at 42 days (Fig. 1C).

**Food intake, growth performance and physical indices**

The cortisol treatments equally suppressed feeding throughout the cortisol and recovery periods (Fig. 2A). On average, over the 42-day experiment and relative to the time-matched Sham treatments, the Cort-I and -II treatments decreased food intake by 43 and 53% respectively. Exogenous cortisol significantly reduced mass gained in a dose-dependent manner (Fig. 2B). Although there was no difference in initial BW between any of the groups (Sham: 195.3 ± 6.3 g; Cort-I: 188.8 ± 5.2 g; Cort-II: 192.1 ± 4.1 g), by the end of the 42-day trial the Sham fish had nearly doubled their mass while the Cort-I and -II fish increased BW by only 50 and 15% respectively. Relative to the Sham groups, the Cort-I and -II treatments elicited sustained dose-dependent reductions in SGR throughout the cortisol and recovery periods, but the growth-suppressing effects of cortisol only reached statistical significance in the Cort-II treatment (Fig. 2C). On average, over the 42-day experiment and relative to the time-matched Sham treatments, the Cort-I and -II treatments decreased SGR by 43 and 70% respectively. Over the course of the experiment, fork length increased by 17.9 and 7.2% in the Sham and Cort-I treatments, respectively, and did not change in the Cort-II treatment (Table 2). While CF increased over the 42-day trial in the Sham fish,
it remained unchanged in the cortisol-treated fish. HSI remained constant in the Sham treatment over the 42-day trial. Conversely, relative to the sham group, HSI decreased by 28% in the Cort-II fish by 28 days and was reduced by 24 and 27% in the Cort-I and -II treatments at 42 days respectively.

Expression of selected genes involved in food intake regulation

Hypothalamic npy, agp, pomc-a2, cocaine- and amphetamine-regulated transcript (cart), POA crf and liver lep-a1 mRNA levels remained unchanged over the course of the experiment in the Sham treatment (Fig. 3). Relative to Sham, npy gene expression were 2.1- and 3.5-fold higher in the Cort-I and -II treatments at 14 days, remained elevated in the Cort-II treatment at 28 days, and returned to basal levels in both cortisol treatments at 42 days (Fig. 3A). Although the agp mRNA levels did not differ between the Sham and Cort-I treatments, agp gene expression was 68% lower in the Cort-II treatment than in Sham at 14 days and recovered thereafter to control levels (Fig. 3B). Hypothalamic pomc-a2 and cart gene expression in the Cort-I and -II treatments did not differ from the Sham treatment (Fig. 3C and D). POA crf mRNA levels in the Cort-II treatment were more than twofold higher than control levels throughout the experiment, and did not differ between the Cort-I and Sham treatments (Fig. 3E). In the Cort-I treatment, liver lep-a1 mRNA levels were 3.1-fold higher than Sham at 14 days but did not differ from control levels thereafter (Fig. 3F). In contrast, lep-a1 mRNA levels in the Cort-II treatment were 4.1- and 8.6-fold higher than Sham at 14 and 28 days respectively, and remained 2.4-fold higher than control levels at 42 days (Fig. 3F).

Expression of leptin in isolated hepatocytes

In the cortisol treatment, hepatocyte lep-a1 mRNA levels were 2.3-fold higher than in the control treatment (Fig. 4). In the combination group, the cortisol-mediated increase in lep-a1 gene expression was abolished by RU486.

Expression of selected genes and hormones involved in growth regulation

Although significant main treatment effects were observed for pituitary gh1 mRNA levels (P=0.002), pituitary gh2 mRNA levels (P<0.001) and plasma GH levels (P=0.007), higher variability for these parameters resulted in few post-hoc treatment-specific differences (Fig. 5). In general, each cortisol treatment had similar effects on pituitary gh1 and gh2 gene expression (Fig. 5A and B). Relative to the sham treatment, the cortisol treatments were associated with higher pituitary gh gene expression at 14 days, higher and lower pituitary gh mRNA levels in the Cort-I and -II treatments at 28 days, respectively, and a return to basal conditions at 42 days. In contrast, the cortisol treatments
values were determined by a two factor ANOVA and Holm-Sidak relative to the Sham 14-day treatment. Statistical differences between the cortisol treatments is indicated by † (P < 0.05). The 42-day fish were sampled 8 days after the osmotic pumps ran out.

were generally associated with a sustained reduction in plasma GH levels (Fig. 5C). While liver ghr2 gene expression did not differ between the Sham and Cort-I treatments, ghr2 mRNA levels were 1.9- and 4.2-fold higher in the Cort-II treatment than in Sham at 14 and 28 days, respectively, and recovered thereafter to control levels (Fig. 5D).

The cortisol treatments also elicited 1.9- to 3.2-fold dose-dependent increases in liver igf1 mRNA levels through the first 28 days of the experiment followed by a return to sham levels at 42 days (Fig. 6A). However, plasma IGF1 levels did not differ between the sham- and cortisol-treated fish during the exogenous cortisol delivery period and were 60% lower in the Cort-II treatment than

Table 2 Fork length, condition factor and hepatosomatic index (HSI) of rainbow trout implanted with either vehicle-(Sham) or cortisol-filled (Cort-I and -II) micro-osmotic pumps for 14, 28 and 42 days

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Time (days)</th>
</tr>
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<tbody>
<tr>
<td>Fork length (cm)</td>
<td></td>
<td>14</td>
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<tr>
<td>Sham</td>
<td>25.4 ± 0.5^A</td>
<td>26.4 ± 0.4^a</td>
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<tr>
<td>Cort-I</td>
<td>24.2 ± 0.4^a</td>
<td>25.3 ± 0.6^a</td>
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<tr>
<td>Cort-II</td>
<td>24.5 ± 0.6^a</td>
<td>24.5 ± 0.4^a</td>
</tr>
<tr>
<td>Condition factor (g/cm³)</td>
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<tr>
<td>Sham</td>
<td>1.38 ± 0.03</td>
<td>1.61 ± 0.03^B</td>
</tr>
<tr>
<td>Cort-I</td>
<td>1.41 ± 0.07</td>
<td>1.41 ± 0.04^a</td>
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<td>Cort-II</td>
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<td>HSI (%)</td>
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<tr>
<td>Cort-II</td>
<td>1.62 ± 0.12^a</td>
<td>1.37 ± 0.13^a</td>
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Values are means ± S.E.M. (n=8). Statistical differences between values were determined by a two factor ANOVA and Holm-Sidak post-hoc test: fork length (treatment: P<0.001, time: P<0.011, treatment×time: P=0.203), condition factor (treatment: P=0.079, time: P=0.026, treatment×time: P=0.15) and HSI (treatment: P=0.017, time: P=0.037, treatment×time: P=0.147). Values for a given parameter and treatment that do not share a common letter are different from one another. At a given time, a difference from the Sham treatment is indicated by * and a difference between the cortisol treatments is indicated by † (P<0.05). The 42-day fish were sampled 8 days after the osmotic pumps ran out.

Figure 3 Brain hypothalamic area (A) neuropeptide Y (npy), (B) agouti-related protein (agrp), (C) pro-opiomelanocortin A2 (pomc-a2), (D) cocaine- and amphetamine-regulated transcript (cart), and (E) preoptic area corticotropin-releasing factor (crf), and (F) liver leptin-A1 (lep-a1) mRNA expression in rainbow trout implanted intraperitoneally with a micro-osmotic pump containing either vehicle (Sham), 20 μg (Cort-I) or 40 μg cortisol/g BW (Cort-II). Cortisol treatments terminated at 34 day as indicated by the dashed line. The mRNA expression values are normalized with elongation factor 1α (ef1α) and the expression ratios are presented relative to the Sham 14-day treatment. Statistical differences between values were determined by a two factor ANOVA and Holm-Sidak post-hoc test: npy (treatment: P<0.001, time: P<0.001, treatment×time: P=0.253), agrp (treatment: P=0.004, time: P=0.115, treatment×time: P=0.418), pomc-a2 (treatment: P=0.826, time: P=0.943, treatment×time: P=0.994), cart (treatment: P=0.0319, time: P=0.875, treatment×time: P=0.441), crf (treatment: P<0.001, time: P=0.107, treatment×time: P=0.485) and lep-a1 (treatment: P<0.001, time: P=0.012, treatment×time: P=0.102) mRNA expression. Bars for a given parameter and treatment that do not share a common letter are different from one another. At a given time, a difference from the Sham treatment is indicated by * and a difference between the cortisol treatments is indicated by † (P<0.05). Values are means ± S.E.M. (n=8).
in Sham at 42 days (Fig. 6B). Relative to the Sham treatment, the cortisol treatments were associated with 1.4- to 2.0-fold increases in liver igfbp1 and igfbp2 mRNA levels at 14 days, 1.4- to 2.0-fold increases in igfbp1 gene expression at 28 days and a 1.9-fold increase in igfbp1 gene expression in the Cort-II treatment at 42 days (Fig. 6C and D).

Overall, throughout the 42-day experiment, we observed a relatively weak positive correlation between the circulating levels of IGF1 and SGR in the sham treatment (R=0.541, P=0.01, data not shown). During the cortisol dosing period, a similar correlation was observed between plasma IGF1 and SGR in the Cort-I treatment (R=0.552, P=0.06), but there was no relationship between these two parameters in the Cort-II treatment (R=-0.008, P=0.98). In contrast, the recovery phase at 42 days was characterized by stronger positive correlations between SGR and plasma IGF1 levels in the Cort-I (R=0.849, P=0.03) and Cort-II (R=0.856, P=0.03) treatments.

Plasma and tissue metabolite levels

Plasma glucose levels in the sham treatment remained unchanged throughout the experiment (Fig. 7A). In the Cort-I treatment, plasma glucose concentrations did not differ from those in the Sham. In contrast, glucose levels in
the Cort-II treatment were 50 and 46% higher than in the Sham at 14 and 28 days respectively. At 42 days, the cortisol-treated fish had plasma glucose levels that did not differ from those in the sham-treated fish. At the tissue level, liver (Fig. 7B) and muscle (Fig. 7C) glycogen content remained unchanged in the Sham fish over the course of the experiment. In general, the cortisol treatments elicited dose-dependent decreases in liver glycogen at 14 and 28 days, but only the 80% reduction in the Cort-II treatment at 28 days reached statistical significance. At 42 days, liver glycogen content in the Cort-I and -II treatments were still 57% lower than in the Sham treatment. In contrast, the cortisol treatments did not affect white muscle glycogen content. In the liver, both cortisol treatments reduced lipid content by 72% at 14 days (Fig. 7D). Although liver lipid content remained low in the cortisol-treated fish throughout the experiment, the concentration of this metabolite decreased over time in the Sham treatment such that the cortisol treatments no longer differed from the Sham at 28 and 42 days. In white muscle, while lipid content in the sham treatment remained unchanged over 42 days and the Cort-I treatment had no effect, the Cort-II treatment reduced lipid stores by 50% relative to Sham at 28 and 42 days but the difference only reached statistical significance at 28 days (Fig. 7E).

**Discussion**

It is generally accepted that chronic stress reduces growth in fish primarily through the actions of cortisol but the specific mechanisms by which chronic cortisol exposure inhibits growth remain poorly understood. In this study, we show that chronic exposure to moderate cortisol levels suppress food intake and provide original evidence implicating liver leptin, POA CRF and hypothalamic AgRP as potential mediators of the appetite-suppressing effects of cortisol in rainbow trout. Moreover, we demonstrate that the growth-inhibiting effects of chronic cortisol exposure are associated with complex adjustments within the GH/IGF axis and a mobilization of fuel reserves. Specifically, our findings indicate that hepatic IGFBP1 plays an important role in reducing the growth-promoting effects of IGF1 during both cortisol exposure and recovery.

Rainbow trout in this study were exposed to physiological cortisol levels averaging ~70 and ~116 ng/ml for a 34-day period. While fish can habituate to some chronic stressors, sustained plasma cortisol levels similar to those used in this study have been observed in response to diverse stressors of anthropogenic and natural origins. For example, chronic exposure to aluminum, copper,

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**Figure 6**

(A) Liver insulin-like growth factor 1 (igf1) mRNA expression, (B) plasma IGF1, (C) IGFBP1 mRNA expression and (D) igfbp2 mRNA expression in rainbow trout implanted intraperitoneally with a micro-osmotic pump containing either vehicle (Sham), 20 μg (Cort-I) or 40 μg cortisol/g BW (Cort-II). Cortisol treatments terminated at 34 days as indicated by the dashed line. The mRNA expression values are normalized with elongation factor 1α (ef1α) and the expression ratios are presented relative to the Sham 14-day treatment. Statistical differences between values were determined by a two factor ANOVA and Holm-Sidak post-hoc test: igf1 (treatment: P < 0.001, time: P = 0.006, treatment × time: P = 0.048), plasma IGF1 (treatment: P = 0.148, time: P = 0.719, treatment × time: P = 0.003), igfbp1 (treatment: P < 0.001, time: P = 0.055, treatment × time: P = 0.692) and igfbp2 (treatment: P = 0.002, time: P = 0.002, treatment × time: P = 0.12). Bars for a given parameter and treatment that do not share a common letter are different from one another. At a given time, a difference from the Sham treatment is indicated by * and a difference between the cortisol treatments is indicated by † (P < 0.05). Values are means ± S.E.M. (n = 8).
selenium or low environmental pH can result in prolonged elevations in plasma cortisol levels (Craig et al., 2009, Wiseman et al., 2011, Kennedy & Picard, 2012, Grassie et al., 2013). In salmonids, sustained increases in cortisol levels are also associated with social subordination (Gilmour et al., 2005) and with specific phases of the life cycle such as smolting (Nilsen et al., 2008) and sexual maturation (Fuzzen et al., 2011). In fact, plasma cortisol levels can reach several hundred ng/ml and remain elevated for weeks to months in sexually mature and migrating Pacific salmon (Carruth et al., 2000, Hinch et al., 2006, Westring et al., 2008). As such, our findings contribute to our understanding of the mechanisms underlying the dynamic changes in food intake, growth and energy mobilization that characterize various chronic stressors and life history transitions.

Consistent with the negative feedback effects of glucocorticoids on pituitary pomc expression and plasma ACTH levels in other fish species (Pickering et al., 1987, Rotllant et al., 2001, Karsi et al., 2005), we show that moderate sustained increases in cortisol levels can chronically decrease pituitary pomc-a2 expression and plasma ACTH levels in rainbow trout. Moreover, we provide novel evidence that the negative feedback effects of cortisol on plasma ACTH can persist for at least 8 days beyond the return of cortisol to baseline levels. In contrast, although cortisol has been shown to decrease POA crf gene expression in a few fish species (reviewed by Bernier et al., 2009), and NPO CRF immunoreactive neurons co-express GRs in rainbow trout (Teitsma et al., 1998), chronically elevated cortisol levels in this study were associated with a sustained increase in POA crf mRNA levels. Similarly, suppression of GR signaling in rainbow trout using the GR antagonist RU486 reduces POA crf mRNA levels (Doyon et al., 2006, Alderman et al., 2012). Together, these results suggest that cortisol has a positive feedback effects on POA crf gene expression in rainbow trout.

The marked reductions in food intake elicited by both cortisol treatments throughout the dosage period show that cortisol is a potent anorexigenic factor in rainbow trout. Similarly, chronic plasma cortisol elevations have previously been shown to suppress feeding in rainbow trout (Gregory & Wood, 1999), channel catfish (Peterson & Small, 2005) and sea bass (Dicentrarchus labrax; Leal et al., 2011), although the cortisol dosages used in these studies resulted in plasma cortisol levels that were at least two-times higher than those of the Cort-II treatment. In goldfish, chronic treatment with high plasma cortisol levels (~270 ng/ml) also inhibited food intake, but lower levels (~50 ng/ml) had a stimulatory effect...
(Bernier et al. 2004). Comparison of our previous results in goldfish to those obtained in this study where plasma levels of ~70 ng/ml effectively halved food intake suggest marked species differences in the actions of cortisol on the regulation of food intake in fish. The sustained inhibitory effects of both cortisol treatments on food intake during recovery also provide novel evidence that cortisol can have a lasting inhibitory effect on appetite in rainbow trout.

The cortisol-induced changes in gene expression of appetite-regulating signals observed in this study suggest that several factors mediate the appetite-suppressing effects associated with chronic plasma cortisol elevations in rainbow trout. Consistent with the stimulatory effects of glucocorticoids on the expression and secretion of leptin from adipocytes in mammals (Lee & Fried 2009), our in vitro results demonstrate that physiological concentrations of cortisol directly stimulate lep-a1 mRNA levels through GRs in rainbow trout hepatocytes. Given the anorexigenic effects of leptin (Murashita et al. 2008, Aguilar et al. 2010, MacDonald et al. 2014) and CRF (Bernier 2006, Ortega et al. 2013) in rainbow trout, the inverse relationship between the expression of these genes and food intake during the dosage and recovery periods suggest that liver leptin and POA CRF mediate at least a portion of the appetite-suppressing effects of cortisol in this species. Whether the cortisol-mediated increase in hepatic lep-a1 gene expression translates into an increase in circulating leptin levels, and whether leptin stimulates CRF release in fish as it does in mammals (Roubos et al. 2012) remain to be determined. The cortisol-induced reduction in the expression of the orexigenic signal AgRP (Cerdá-Reverter et al. 2011) suggest that this hypothalamic factor may also contribute to the anorexie effects of cortisol. In contrast, we found no evidence that the anorexigenic POMC and CART neurons of the hypothalamus contribute to the cortisol feeding response. Although the cortisol treatments in this study stimulated the hypothalamic gene expression of the orexigenic factor NPY as in goldfish (Bernier et al. 2004), clearly the current experimental conditions favored an anorexic response over an orexigenic one. Finally, since high plasma glucose and fatty acid levels inhibit food intake in rainbow trout and there is evidence that hypothalamic glucosensing and fatty acid-sensing systems are involved in regulating the expression of appetite-regulating genes in this species (Polakof et al. 2011, Librán-Pérez et al. 2012), we suggest that cortisol may inhibit food intake indirectly via its hyperglycemic and lipolytic effects. In fact, as observed in this study, rainbow trout reared under stressful conditions induced by high stocking density and made hyperglycemic are characterized by elevated hypothalamic CRF and NPY mRNA levels (Conde-Sierra et al. 2010).

Our results concur with previous studies in rainbow trout (Barton et al. 1987, Gregory & Wood 1998, De Boeck et al. 2001) and other fish species (e.g. Bernier et al. 2004, Peterson & Small 2005, Leal et al. 2011) showing that chronically elevated cortisol levels inhibit growth. In addition, our results demonstrate that the factors contributing to the growth-suppressing effects of cortisol are differentially recruited according to plasma cortisol levels. The fact that Cort-II inhibited mass gained, fork length and CF to a greater extent than Cort-I despite having similar effects on food intake suggests that the relative contribution of reduced food intake to the growth-suppressing effects of cortisol is superseded by factors that reduce feed conversion efficiency when plasma cortisol levels increase above ~70 ng/ml in rainbow trout. In fish chronically treated with cortisol, lower feed conversion efficiencies may result from a reduction in nutrient absorption in the gut, an increase in metabolic rate, a mobilization of fuel reserves and an inhibition of the growth-promoting effects of the GH/IGF axis (Davis et al. 1985, Barton et al. 1987, Gregory & Wood 1999, Mommens et al. 1999, De Boeck et al. 2001, Small et al. 2006).

In this study, chronic cortisol had opposite effects on GH production and circulating levels. Consistent with the presence of glucocorticoid response elements in the rainbow trout gh1 and gh2 promoter (Yang et al. 1997), we show that chronic cortisol can stimulate pituitary gh1 and gh2 transcription but the effects are complex, dependent on both exposure duration and dosage, and unexpectedly associated with a reduction in plasma GH levels. In general, cortisol and stressors have equivocal effects on plasma GH levels in teleosts. In salmonids, whereas acute stressors either have no effect or decrease plasma GH levels, chronic stressors can increase circulating GH levels (Pickering et al. 1991, McCormick et al. 1998, Wilkinson et al. 2006, Shepherd et al. 2011, Nakano et al. 2013). Given the complexity of the neuroendocrine control of GH secretion (Canosa et al. 2007), the inhibitory effects of cortisol on the plasma levels of the GH secretagogue ghrelin (Pankhurst et al. 2008, Janzen et al. 2012) and the presence of GH binding proteins in fish (Sohn et al. 1998), we suggest that the discrepancy between pituitary GH gene transcription and plasma GH levels in this study may result from changes in the multifactorial regulation of pituitary GH secretion and from an increase in the circulating levels of GH binding
proteins. Overall, even though IGFs are recognized as the primary mediators of the growth-promoting effects of the GH/IGF system, since GH can directly stimulate muscle growth in fish (reviewed by Fuentes et al. (2013)) our results indicate that a reduction in plasma GH levels may contribute to the growth-suppressing effects of chronic cortisol in rainbow trout.

In the liver, chronic cortisol exposure resulted in an increase in the expression of several key effectors of the GH/IGF system which together enhanced GH signaling while simultaneously reducing the role of IGF1 as a growth-promoting agent. Consistent with previous observations in salmonids (Pierce et al. 2005, Reindl & Sheridan 2012) and tilapia (Oreochromis mossambicus; Pierce et al. 2012) cortisol upregulated the expression of hepatic ghr2 in this study. Chronic cortisol exposure also elicited dose-dependent increases in hepatic igf1 expression that closely matched the increase in ghr2 mRNA levels, results which suggest an increase in hepatic GH sensitivity. However, despite eliciting a two- to threefold increase in hepatic igf1 expression, the cortisol treatments did not affect plasma IGF1 levels. Instead, the cortisol-induced increases in hepatic igf1 gene expression were paralleled by changes in liver igfbp1 and igfbp2 mRNA levels after 14 days and by an up-regulation of igfbp1 expression after 28 days. Since over-expression of low molecular weight IGFBPs can inhibit growth in mammals and fish (reviewed by Duan et al. 2010), we suggest that the cortisol-induced increases in hepatic igfbp1 and igfbp2 expression reduced the role of IGF1 as a growth-promoting agent and contributed to the discordant relationship between plasma IGF1 levels and SGR in the Cort-II treatment. In contrast, channel catfish fed cortisol diets that raised plasma cortisol levels to ~180 ng/ml daily for 4 weeks were characterized by reduced hepatic ghr expression, no change in liver igf1 mRNA levels, a marked increase in the circulating levels of a low molecular weight IGFBP and depressed plasma IGF1 levels (Peterson & Small 2005, Small et al. 2006). While differences in dosage, modes of application, and species-specific transcriptional regulation of the GH/IGF system may explain the variable effects of chronic cortisol on liver ghr and igf1 gene expression between studies, our results suggest that an increase in the circulating levels of low molecular weight IGFBPs is a conserved mechanism by which chronic stress inhibits growth in fish.

Eight days post cortisol delivery, the transcriptional activity of the GH/IGF system and the plasma levels of GH and IGF1 indicate that while pituitary GH production and hepatic GH signaling are largely recovered, the growth-promoting actions of IGF1 are still blunted. As observed during the cortisol dosing period, the parallel changes in hepatic ghr2 and igf1 mRNA levels during recovery suggests a cause-and-effect relationship between these elements of the GH/IGF axis. In the Cort-II treatment, the reduced plasma IGF1 concentration and increased hepatic igfbp1 gene expression likely contributed to the sustained growth inhibition. Fasting also increases the circulating levels of low molecular weight IGFBPs in fish (Kelley et al. 2001, Peterson & Small 2004) and in fasted channel catfish the expression of hepatic igfbp1 can remain elevated even after 15 days of refeeding (Peterson & Waldbieser 2009). Therefore, beyond the direct actions of cortisol on the transcriptional regulation of igfbp1, it is possible that the nutritional status of the cortisol-treated fish contributed to the sustained elevation in liver igfbp1 expression observed during recovery.

Consistent with its key role in regulating energy mobilization during stress, our results implicate cortisol in the catabolism of carbohydrates and lipids. The cortisol-mediated changes in HSI, CF, as well as in liver and muscle glycogen and lipid content, also demonstrate that the catabolic effects of cortisol in this study had a much larger impact on the energy reserves of the liver than on those of white muscle. In the liver, numerous fish studies have shown that cortisol increases gluconeogenic capacity (Mommsen et al. 1999, Aluru & Vijayan 2007, Momoda et al. 2007) and the increases in plasma glucose levels associated with the Cort-II treatment during the dosing period support these findings. The marked reductions in liver glycogen content also suggest that glycogenolysis may have contributed to the cortisol-induced increase in plasma glucose levels. However, whether cortisol has a direct glycolytic effect on the liver is equivocal. Previous studies in fish have shown that cortisol exposure can increase, decrease, or have no effect on liver glycogen content and there is generally no consensus as to the role of cortisol in liver glycogen metabolism (Mommsen et al. 1999, De Boeck et al. 2001, Laiz-Carrion et al. 2003). In tilapia (O. mossambicus), while injections of both cortisol and leptin increase plasma glucose levels, only leptin decreases liver glycogen content (Baltzegar et al. 2014). Given our observation that cortisol stimulates leptin gene expression, an interesting avenue for future research will be to determine whether the glycolytic effects of cortisol in rainbow trout are indirect and mediated by leptin. The reduction in liver and muscle lipid content in the cortisol-treated fish is consistent with the increased lypolytic capacity of chronically stressed fish (Mommsen et al. 1999). Cortisol may deplete lipid reverses in fish by increasing the activity of various lipases.
(Sheridan 1986, Baltzegar et al. 2014) and glycerol utilization (Vijayan et al. 1991), and by reducing the lipogenic potential of the liver (Vijayan et al. 1990, Laiz-Carrion et al. 2003, Lopez-Patino et al. 2014). Moreover, given the known lipolytic properties of GH in the liver (Bjornsson et al. 2002), our results suggest that cortisol may also promote lipolysis via its stimulatory effects on liver gh2r expression.

Results from this study provide a novel perspective on the multiple interacting pathways by which cortisol contributes to the coordination of energy mobilization and expenditure during chronic stress in fish. Together our findings show that the growth-suppressing effects of chronic cortisol in rainbow trout result from a sustained reduction in food intake, complex transcriptional changes in the GH/IGF1 axis that reduce the growth-promoting actions of GH and IGF1 while enhancing the effects of GH on the liver, and from a mobilization of carbohydrate and lipid reserves. Specifically, our results suggest that liver leptin and POA CRF are mediators of the anorexigenic effect of chronic cortisol, and that hepatic IGFBP1 likely plays a key role in suppressing the growth-promoting action of IGF1. Finally, our results highlight the need to further define the individual and synergistic actions of cortisol, GH and leptin in the mobilization of energy during chronic stress, as well as the possible indirect actions of cortisol on the regulation of food intake and the GH/IGF1 axis mediated through changes in the levels of circulating metabolites.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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