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Extended fasting does not affect the liver innate immune response in rainbow trout

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ABSTRACT

Activation of immune response pathway is energy demanding. We tested the hypothesis that negative energy balance will curtail the liver's capacity to evoke an immune response in rainbow trout (*Oncorhynchus mykiss*). Fish were either fed or fasted for 118 d and challenged with lipopolysaccharide (LPS) to determine the liver capacity to elicit an immune response. Fasting led to negative specific growth rate, reduced tissue metabolite levels, and higher transcript abundance of SOCS-2. LPS treatment increased the liver transcript abundances of IL-1β and IL-8 and serum amyloid protein A, while SOCS-2 was reduced. LPS lowered plasma cortisol level only in the fasted fish, but did not affect liver glucocorticoid or mineralocorticoid receptor protein expressions. Extended fasting did not suppress the liver capacity to evoke an immune response. Upregulation of liver SOCS-2 may be playing a key role in the energy repartitioning, thereby facilitating immune response activation despite extended fasting in trout.

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1. Introduction

Prolonged fasting in fishes results in metabolic suppression and is associated with reduced plasma glucose, liver glycogen and liver metabolic capacity (Foster and Moon, 1991; Vijayan and Moon, 1992; Navarro and Gutiérrez, 1995). The liver plays an important role in the intermediary metabolism and is a key energy target for hormone action. We previously demonstrated the ability of this tissue to express immune response mediators in response to endotoxin shock, suggesting a role for this tissue in the inflammatory response (Philip et al., 2012). Even though conditions of fasting are well tolerated by most fish species, dietary restriction may be deleterious with respect to immune function (Martin et al., 2010).

Fish innate immune responses involve the synthesis of pro-inflammatory cytokines, including IL-1β and IL-8, and acute phase proteins (APP), including serum amyloid protein A (SAA) (Uribe et al., 2011). Pro-inflammatory cytokines are expressed in salmonids during the early phases of an infection, while APPs are synthesized in the liver in response to pro-inflammatory cytokines to aid in pathogen elimination (SaranyaRevathy et al., 2012). Mounting an immune response requires energy for protein synthesis, leading to increase in metabolic activity (Martin et al., 2010). Dietary restrictions lead to reduced growth and heightened stress response which in turn impact the effectiveness of a robust immune response (Liu et al., 2013; Martin et al., 2010). The impact of these responses has been shown to differ depending on duration of feed restriction, pathogen dynamics as well as species-specific evolutionary adaptations to seasonal changes in food availability (Liu et al., 2013).

Feed deprivation for a week leads to decreased mortality rates to *Edwardsiella ictaluri* and higher mortality to *Flavobacterium columnare* in channel catfish (Shoemaker et al., 2003; Wise et al., 2008). Fasting for 28 days reduced mRNA abundance of immune-related genes in the liver of Atlantic salmon (*Salmo salar*), while fasting for 31 days decreases non-specific immune parameters, including haemagglutinating activity and respiratory burst activity in the sea bass (*Dicentrarchus labrax*) and blackspot sea bream (*Pagellus bogaraveo*) (Martin et al., 2010; Caruso et al., 2011). Long-

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term fasting in anadromous Arctic charr (*Salvelinus alpinus*), a salmonid with long term fasting as a part of their life history strategy, downregulated cytokine response to LPS stimulation, thought to be an adaptation to conserve energy resources (Philip et al., 2014). The cortisol response to food deprivation has been equivocal and this may be due to various factors, including species differences, duration of fasting and the life history of the animal (Vijayan and Moon, 1992; Pottinger et al., 2003; Vijayan et al., 2010). Though it is clear that nutritional perturbations can modulate immune responses in teleosts, the molecular mechanisms for this are largely unknown.

The suppressors of cytokine signalling (SOCS) are key modulators of the immune response and are affected by fasting (Philip et al., 2014). Even though they function as negative regulators of cytokine signalling, they are also known to regulate diverse physiological functions, including growth and development in mammals (Croker et al., 2008; Kile and Alexander, 2001). Homologues of all the eight mammalian SOCS family members have been discovered in fishes, with many of them having multiple copies in fish (Wang et al., 2011). SOCS-1-3 have been characterized in salmonids (Wang and Secombes, 2008), but their functional roles are just beginning to emerge. It has been shown that SOCS affect cytokine and growth hormone (GH) signalling by acting as negative regulators of the JAK/STAT pathway (Croker et al., 2008). Increased circulating cortisol levels in response to stress upregulate SOCS-1 and SOCS-2 expression in rainbow trout liver, suggesting a role for the SOCS in regulating stress-immune interactions in fish (Philip et al., 2012). Also a recent study suggested a possible adaptive role associated with SOCS regulation in the liver during natural fasting in anadromous Arctic charr (Jørgensen et al., 2013; Philip et al., 2014). While the Arctic charr resorts to voluntary long-term fasting as a life-strategy, other salmonids, including rainbow trout (*Oncorhynchus mykiss*) do not exhibit natural fasting as a life-history strategy to the same extent as in Arctic charr. Consequently, rainbow trout is an appropriate model to test the long-term effect of nutrient deprivation on SOCS and cytokine regulation and its implication in energy substrate partitioning.

Here we tested the hypothesis that fasting will upregulate transcript levels of SOCS, a negative regulator of growth, and suppress innate immune response in rainbow trout liver. Recent studies clearly implicate liver as an immune-responsive tissue in trout, in addition to being a key tissue in intermediary metabolism (Philip et al., 2012, 2014). Fish were fed or fasted for 118 d and injected with LPS, a well-established immunostimulant (Engelsma, 2002; MacKenzie et al., 2006) and sampled at 8, 72 and 98 h post-injection. Plasma cortisol, glucose and lactate levels, liver glycogen content, protein expressions of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) were measured as markers of the putative stress response. We also assessed inflammatory response in the liver by measuring the mRNA abundances of pro-inflammatory cytokines, IL-1β and IL-8 as well as the three isoforms of SOCS, SOCS-1, SOCS-2 and SOCS-3, while SAA was used as an indicator of the acute phase response.

2. Materials and methods

2.1. Fish

Juvenile rainbow trout (55 ± 10 g body mass) were obtained from the Washington state Klickitat Hatchery, Glenwood, WA, USA and maintained at the USGS Columbia River Research Laboratory, Cook, WA, USA, at 6–7 °C on a 12:12-h light/dark cycle. Fish were fed daily to satiety with commercial dry pellet till the start of the experiment.

2.2. Experimental design

On 10 January 2012, rainbow trout were weighed, fork length measured, tagged with passive-integrated transponder (PIT) tags, which allowed us to track growth of individual fish. Fish were then distributed randomly into four tanks (n = 20 per tank). Rainbow trout in two of the tanks were fed as stated before, while the rainbow trout in the other two tanks were fasted until 7 May 2012 (118 days). On 7 May, the fish body mass (BM) and fork length (FL) were measured and used to calculate the condition factor (CF; BM/FL^3 x 100). Specific growth rate (SGR) was calculated by the formula $[(\ln BM_T - \ln BM_t/T\text{-}t) \times 100]$ where BM_T and BM_t are the weights of the fish in May and January, respectively, and T-t the number of days between weight measurements. On the same day, half the fish in each tank were injected with LPS [(*Escherichia coli*, 0111:B4; Sigma; 2.5 mg/kg wt in saline) and the other half with the saline vehicle and redistributed into four different tanks (fed and LPS injected, n = 20; fed and saline injected, n = 20; fasted and LPS injected, n = 20; fasted and saline injected, n = 20). All fish were food-deprived after injection and sampled (n = 6–8) at 8, 72 and 98 h post-injection. Fish were euthanized with an overdose of neutralized MS222 and blood samples were immediately centrifuged at 5000x g for 5 min. Plasma was separated and stored at −80 °C to measure cortisol, glucose and lactate levels later. Liver tissues were stored at −80 °C for glycogen, transcript and protein expression analyses later. All analyses were completed within a year of sampling.

2.3. Plasma cortisol, glucose, lactate levels and liver glycogen content

Plasma cortisol levels were measured using a $[^3H]$-labeled cortisol radioimmunoassay as described previously (Alsop et al., 2009). Plasma glucose levels were measured by NAD reduction with hexokinase (HK)-glucose-6-phosphate dehydrogenase (G6PDH) in Tris buffer (120 mM Tris-base, 80 mM Tris-HCl, 5 mM NAD, 2 mM MgS04, 5 mM ATP). The reaction was started with G6PDH (0.4 U/ml) and HK (0.5 U/ml). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA). The same protocol was used to determine liver glycogen content by analysing liver glucose before and after amyloglucosidase hydrolysis. Plasma lactate levels were measured by monitoring NAD reduction with lactate dehydrogenase (LDH) in hydrazine buffer (0.2 M; pH 9.5). The reaction was started with LDH (10U/well). The reaction was measured at 22 °C by continuous spectrophotometry at 340 nm using a microplate reader (VersaMax; Molecular Devices Corp., Palo Alto, CA, USA).

2.4. SDS-PAGE and immunodetection

Sample protein concentrations were measured with bicinchoninic acid (BCA) reagents using bovine serum albumin as the standard. SDS–PAGE and immunodetection were carried out as described before (Boone and Vijayan, 2002). Briefly, sample protein (40 μg protein per lane) was separated on 10% polyacrylamide gels and the proteins were transferred onto nitrocellulose membranes. The membranes were probed with either rabbit polyclonal anti-trout GR (1:1000; Sathiya and Vijayan, 2003) or rabbit polyclonal anti-trout MR (1: 1000; Jeffrey et al., 2012). The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000; BioRad, Hercules, CA, USA). Protein bands were detected using chemiluminescence reagent, ECL plus (Amersham Biosciences, Piscataway, NJ, USA), and the molecular mass confirmed with Precision Plus molecular weight markers (BioRad). The bands

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