Comparative Biochemistry and Physiology, Part D 13 (2015) 52–59

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Differential metabolite levels in response to spawning-induced inappetence in Atlantic salmon *Salmo salar*

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ARTICLE INFO

Article history:
Received 14 October 2014
Received in revised form 5 January 2015
Accepted 5 January 2015
Available online 10 January 2015

Keywords:
Atlantic salmon
Inappetence
Metabolomics
Salmo salar
Serum

ABSTRACT

Atlantic salmon *Salmo salar* undergo months-long inappetence during spawning, but it is not known whether this inappetence is a pathological state or one for which the fish are adapted. Recent work has shown that inappetent whale sharks can exhibit circulating metabolite profiles similar to ketosis known to occur in humans during starvation. In this work, metabolite profiling was used to explore differences in analyte profiles between a cohort of inappetent spawning run Atlantic salmon and captively reared animals that were fed up to and through the time of sampling. The two classes of animals were easily distinguished by their metabolite profiles. The sea-run fish had elevated ω-9 fatty acids relative to the domestic feeding animals, while other fatty acid concentrations were reduced. Sugar alcohols were generally elevated in inappetent animals, suggesting potentially novel metabolic responses or pathways in fish that feature these compounds. Compounds expected to indicate a pathological catabolic state were not more abundant in the sea-run fish, suggesting that the animals, while inappetent, were not stressed in an unnatural way. These findings demonstrate the power of discovery-based metabolomics for exploring biochemistry in poorly understood animal models.

1. Introduction

The salmonid family includes cool temperate anadromous fishes, including many species of important commercial and conservation concern such as Atlantic salmon *Salmo salar*. All salmonid fish experience conditions of extreme physiological change over the course of their lives, including the shift from freshwater to seawater and subsequently back to freshwater for spawning. In addition, there are also extended periods with little to no active feeding during overwintering and during the spawning run. One might generally expect that extended periods of inappetence could have a deleterious impact on an animal, which could in turn be detected via circulating biomarkers; perhaps the most well-known example of this is ketosis and acidosis in humans, which can occur in response to starvation or prolonged fasting. It was recently found that inappetent whale sharks could be distinguished by circulating biomarkers, including markers such as keto-acids that are similar to those expected in human starvation (Dove et al., 2012). However, it is not known if inappetence of Atlantic salmon during migration and spawning could even be attributed to or distinguished by some form of abnormal physiology. It is unclear whether long-term inappetence is an inherently stressful event (and thus potentially dangerous as it could compound the effect of anthropogenic stressors), or whether this is a natural condition that the fish have evolved or adapted to efficiently handle during their regular life cycle. Characterizing and understanding the response of salmonid fish to such an extreme condition may provide insight into the physiology of all fishes and the potential challenges faced by some important endangered populations during their complex life histories. Taken together, these insights may help enable more efficient monitoring of these species, with broader economic impact via the extensive efforts of federal and state wildlife management agencies.

Part of the challenge of exploring these potentially stressful physiological changes over the lives of these fish is in the overall lack of biochemical knowledge about the animals. A common approach in veterinary and husbandry practice is to measure serum or plasma levels of molecules with already-known clinical significance in the specific species under study (Parry, 1961; Jeffries et al., 2011), or molecules with known significance in other model species that are expected to be important in the species under study (Dove et al., 2005, 2010; Hevroy et al., 2012). However, it is generally unknown what the serum levels of a given metabolite (e.g., glucose or free fatty acids) should be in normal versus different diseased or affected states for an arbitrarily-chosen species of fish. To this end, discovery-oriented approaches are particularly valuable to begin to understand the key determinants and identifiers of animal response to different conditions.

There has been increasing use of high-throughput, systems scale ("-omics") technologies to assess the physiological state of salmonids

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http://dx.doi.org/10.1016/j.cbd.2015.01.001
1744-117X/

under different conditions. Functional genomics approaches have studied the transcriptional changes in the liver and other tissues of salmonids (Miller et al., 2009; Martin et al., 2010; Evans et al., 2011). There has also been some recent attention to exploration of salmonid physiology through the systematic measurement of small-molecule biochemical intermediates (metabolites) in the field known as "metabolomics" (Solanky et al., 2005; Viant et al., 2006; Viant, 2007; Lin et al., 2009; Hines et al., 2010). The majority of the focus in such studies has been on the toxicological and environmental impacts on the fish, including petrochemicals (Lin et al., 2009) and pesticides (Viant et al., 2006). While the study of such anthropogenic sources of disturbance to the salmonid life cycle are obviously of value, a better understanding of the salmonid's response to natural life cycle events would help to provide a more detailed picture of salmonid physiology and biochemistry, as well as suggest whether these life cycle events might compound the effects of anthropogenic stressors.

Recently, there was one metabolomics study of a salmonid response to short-term fasting, in juvenile rainbow trout *Oncorhynchus mykiss* (Kullgren et al., 2010). That study used proton nuclear magnetic resonance (NMR) spectroscopy to measure metabolite levels in plasma, liver, and muscle extracts, and found significant changes in lipids and energy metabolites in these samples. However, the authors noted that using hatchery-reared fish under laboratory conditions may limit generalization of the results, as wild fish are usually leaner and may have different responses to fasting. Additionally, the scope and number of analytes studied (due to sensitivity requirements and limitations on analyte identification with an NMR-based approach) was limited. Finally, that study involved a 28-day fasting experiment that did not include the impact of the months-long inappetence that salmonids experience during spawning and overwintering. A subsequent study used mass spectrometry in place of NMR but did not address the other limitations (Baumgarner and Cooper, 2012).

The purpose of this work was to test whether there are indications that long-term inappetence of salmon is a pathological state as in other organisms and to explore the changes in blood metabolite profiles caused by long-term inappetence. These goals are accomplished using a highly sensitive mass spectrometry-based approach to compare differences in blood metabolite levels between the same generation of hatchery-raised and -fed control fish as compared to wild, sea-run fish fasting for 5 to 6 months. Serum samples were taken from each of these corresponding cohorts and then analyzed to identify biochemical indicators of response to inappetence.

2. Materials and methods

2.1. Fish and experimental conditions

The control group was a set of 20 post-spawning adult female domestic *S. salar* housed at the White River National Fish Hatchery (Bethel, VT). These fish spent all 4–5 years at the hatchery and were feeding through spawn in October/November of 2010 and sampling in December of 2010. The fish were fed a maintenance diet of 1.5% body weight one time daily with Corey AquaBrood (Fredericton, New Brunswick, Canada; list of ingredients provided in Supplementary Information). Blood samples were collected post-spawn in December of 2010.

The inappetent group consisted of 20 feral sea-run female *S. salar* that returned to the U.S. Fish and Wildlife Service (USFWS) Richard Cronin National Salmon Station (Sunderland, MA) in May of 2010. These fish originated at the USFWS White River National Fish Hatchery but were released as either non-feeding fry or one-year smolts into the Connecticut River. At age 5, the fish returned to the Connecticut River during the spring (April/May) to spawn but were captured at the Holyoke Dam fish lift (Holyoke, MA) and transported to the Richard Cronin National Salmon Station. The fish were maintained at that facility in non-feeding status until they spawned in November of 2010. These fish stop active feeding at the onset of their spawning migrations and do not feed throughout the entire holding period at the Richard Cronin facility (approximately 5–6 months). Blood samples were collected post-spawn in December of 2010, the day after the control group samples were taken.

Sampling of fish began at 7:30 AM for each group of fish; the control fish had last been fed at 4:00 PM the night before. The average sizes of sampled fish were 78.3 cm and 4.4 kg and were similar between the two groups. The sea-run fish available for sampling were to be reconditioned for feeding and subsequent breeding after this study. Since the hatchery typically has enough sperm available for fertilization, only female kelt are maintained, and so the sea-run fish available for sampling were all female. Accordingly, the domesticated (control) fish selected for sampling were exclusively female to remove any possible gender effects from the study.

2.2. Sampling procedure and sample preparation

Blood was drawn from each fish from its hemal arch using heparinized syringes and stored on ice for 1 h. A Pasteur pipette was inserted into the tube to ring the pre-formed clot and allow the red blood cells to collect at the bottom of the tube overnight at 4 °C; the resulting serum was then transferred to new tubes, and stored at −80 °C until metabolomics analysis. Aliquots of 50 μL of each of these samples were then further processed for mass spectrometry analysis by first using 150 μL of ice-cold acetonitrile to precipitate any protein that may have remained. The sample was then centrifuged at 21,100 *g* for 7 min and the supernatant removed. The resulting serum was then evaporated to dryness using a CentriVap centrifugal concentrator at 40 °C and derivatized following the protocol laid out by Kind et al. (2009). Briefly, the samples were resuspended in 10 μL of 40 mg/mL *O*-methylhydroxylamine hydrochloride in pyridine and shaken at 1400 rpm for 90 min at 30 °C. Then, 90 μL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) + 1% trimethylchlorosilane (TMCS) (Thermo Scientific, Lafayette, CO) was added to the samples, which were then shaken at 1400 rpm for 30 min at 37 °C. Samples were centrifuged at 21,100g for 3 min, and 50 μL of the supernatant was added to an autosampler vial. Samples were spiked with 0.25 μL of a retention time standard solution consisting of fatty acid methyl esters (FAMEs) and an internal standard of nonadecanoic acid methyl ester dissolved in dimethylformamide.

2.3. GCxGC-MS analysis

A LECO Pegasus 4D instrument with an Aglient 7890A gas chromatograph and 7683B autosampler was used to analyze the samples. Complete method details can be found in the supplemental information. Briefly, 1 μL sample volume was injected into the inlet in splitless mode at 250 °C. A constant flow rate of helium was kept at 1 mL/min. The oven temperature program started at 50 °C and ramped to 295 °C over 28 min, with the secondary oven kept at an offset of 15 °C and the modulator temperature offset kept at 35 °C from the main oven. The first column was an HP-5 30 m × 0.320 mmID × 0.25 μm (Agilent, Santa Clara, CA), and the second was Rtx-200 2 m × 0.180 mmID × 0.20 μm (Restek, Bellefonte, PA). Each experimental sample was analyzed with technical duplicate injections, with sample order randomized to limit batch effects. A pooled quality control sample was created from a small aliquot of each sample. This pooled quality control sample was prepared in the same way as the two sets of experimental samples and was injected after every three to six samples to facilitate later data correction. A typical chromatogram is presented in Supplementary Fig. 1.

2.4. Data analysis

Sample runs were first analyzed in the instrument manufacturer's software, ChromaTOF (LECO, St. Joseph, MI), to determine baseline,