Steroids 88 (2014) 26–35

Contents lists available at ScienceDirect

Steroids

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

Identification of cortisol metabolites in the bile of Atlantic cod *Gadus morhua* L.

Alexander P. Scott [a,*], Tim Ellis [a], Helge Tveiten [b]

[a] Centre for Environment, Fisheries and Aquaculture Science, Barrack Road, Weymouth, Dorset DT4 8UB, UK
[b] Nofima Marin, Postboks 6122, 9291 Tromsø, Norway

ARTICLE INFO

Article history:
Received 29 January 2014
Received in revised form 2 April 2014
Accepted 23 May 2014
Available online 10 June 2014

Keywords:
Fish
Steroid metabolism
Bile
Sulfate
Cortisol
Stress

ABSTRACT

Interpretation of plasma cortisol levels in wild-caught fish is confounded by the stress of capture. Measurement of cortisol metabolites in fish bile could provide a method for assessing the stress level of wild fish because the time-lag for metabolism, conjugation and excretion into bile avoids the effects of sampling stress. To determine which biliary metabolite(s) to target, four Atlantic cod, *Gadus morhua* L., were injected with radioactive cortisol. After 22 h, the bile was collected and found to contain 30% of the injected activity. Cortisol metabolites were extracted from diluted bile samples using solid phase extraction and the radioactive metabolites separated by several different chromatographic procedures. The metabolites were predominantly present as sulfates (95%) with the remainder being glucuronidated. Chromatography split the sulfates into at least seven peaks, and acid solvolysis (which removes sulfate groups from steroids) generated four major radioactive steroids. These were identified, using microchemical reactions and re-crystallization to constant specific activity, as: 11β,17,21-trihydroxypregn-4-ene-3,20-dione (cortisol), 3α,11β,17,21-tetrahydroxy-5β-pregnan-20-one (tetrahydrocortisol; THF), 3α,17,21-trihydroxy-5β-pregnane-11,20-dione (tetrahydrocortisone; THE) and 3α,17,20β,21-tetrahydroxy-5β-pregnan-11-one (β-cortolone). The last of these was the most abundant, and thus a likely target for a biliary stress assay. Studies were also carried out to determine the best method for extraction and solvolysis of sulfates. Solid phase extraction (i.e. using octadecylsilane) was found to be too unreliable for routine use. Even though the extraction efficiency could be improved by acidifying the bile, this caused premature solvolysis of sulfated steroids. Acid solvolysis of unextracted bile worked best (*c.* 90% converted to free steroids) on volumes that were 1 μL or lower. Aryl sulfatase digestion of unextracted bile did not work well (only 20% of radioactivity was converted to free steroids).

1. Introduction

There is a growing appreciation that various anthropogenic disturbances in the marine environment (e.g. acoustic surveys, boat noise) may cause stress in wild fish [1,2]. As human uses of marine areas increase (e.g. construction and operation of off-shore windfarms), we need to understand any potential stress effects on wild fish. There is a very well-established means of determining the stress level of fish held under laboratory conditions, i.e. measurement of the level of cortisol in the blood plasma [3]. However, the very attribute of cortisol that makes it the premier indicator of stress in fish – its responsiveness – prevents its use in wild fish sampled from natural environments. Blood cortisol production increases within a few minutes in response to capture stress [4,5], while capture methods such as trawling and long-line fishing often extend over several hours. By the time blood is collected from such fish, pre-capture levels of plasma cortisol levels will be completely hidden by the stress of capture.

The same problem of avoiding capture (and sampling) stress faces scientists working on free-ranging terrestrial vertebrates. However, it has been largely circumvented by measuring levels of the metabolites of cortisol (or corticosterone) in samples of feces [6,7]. Feces can be collected without disturbing, let alone capturing, the animals. Even if it is necessary to capture or disturb the animals, the time delay between cortisol production and the formation and subsequent excretion of its metabolites is sufficiently long (hours rather than minutes) that capture-induced effects can effectively be ignored [8].

Since the hepato-biliary-fecal route is the major route for cortisol clearance in fishes [3,9] as in higher vertebrates, a similar procedure should be feasible in fishes. Initial studies on wild

* Corresponding author. Tel.: +44 01305 206600.
E-mail address: Alexander.scott@cefas.co.uk (A.P. Scott).

http://dx.doi.org/10.1016/j.steroids.2014.05.014
0039-128X/

tropical reef fish have proved promising, with measurable levels of cortisol in feces being related to putative stressors [10–12]. However, collection of feces from free-living wild fish is challenging as it requires observation by scuba-diving, and is therefore restricted in field application to clear, shallow, inshore waters [13].

An alternative potential method for sampled wild fish is to measure cortisol metabolites in bile fluid. This was initially proposed by Pottinger et al. [14] who demonstrated in rainbow trout *Oncorhynchus mykiss* Walbaum, that concentrations of immunoactive cortisol (in the form of glucuronides and/or sulfates) in the bile 24 h after the application of stress were markedly higher than in control samples.

Bile fluid is easy to collect from sampled (dead) fish, and measurement of biliary cortisol metabolites has the advantage over fecal sampling that no further catabolism will have occurred within the gut [15], so it is a step closer to representing plasma cortisol. In developing such a stress assay for wild caught fish, the first question to answer is "What metabolites of cortisol are present in the bile?" The work described in the present paper attempts to answer this question by injecting four Atlantic cod *Gadus morhua* with tritium-labelled cortisol and then characterizing the main radioactive metabolites present in the bile the following day.

2. Materials and methods

2.1. Injection of tritiated cortisol into live fish

The aim of this experiment was to generate tritium-labelled biliary metabolites of cortisol. Tritium-labelled cortisol (hydrocortisone [1,2,6,7-3H]; catalogue No. NET396250UC) in ethanol was purchased from Perkin Elmer (www.perkinelmer.co.uk). This was prepared for injection by evaporation of the ethanol (45 °C, under a nitrogen stream) and re-dissolving in 20 μL ethanol and 1 mL 0.9% saline (microfiltered). Activity was determined by mixing a 5 μL subsample with 7 mL scintillation fluid and counting.

The experiment was carried out at the Aquaculture Research Station in Tromsø, Norway, in November 2012. Hatchery-reared cod (2.5 years old), supplied by the Nofima cod breeding center in Kraknes, Norway, were held at the Research Station for 2 weeks in flow-through tanks under ambient seawater temperature, with an 8:16 light:dark photoperiod and fed a pelleted diet daily. However, the fish were not fed for 5 days prior to use in the experiments and seawater temperature was 6.8 °C.

Individual cod were caught from the holding tanks, anesthetized (MS222, 0.07 g L^{-1}), and injected with approximately 0.23 mL of the labelled cortisol solution: two fish (617 and 805 g) were injected via an intraperitoneal route and two fish (637 and 541 g) via an intramuscular route (ventral to the dorsal fin). The amount of radioactivity injected into each fish was estimated (from a sub-sample of solution) at 1.0 MBq. After injection, each fish was placed in a polythene bag containing 30 L of aerated seawater (no flow-through) which was suspended in a tank with flowing sea water (for temperature control). The fish were swimming normally within 30 min. Water samples (1 mL) were collected from the bags before, and at 1.5 h, 2.5 h and 22 h after introduction of the fish. The fish were removed after 22.5 h, anesthetized (MS222, 0.14 g L^{-1}), blood sampled using a heparinized vacutainer and pithed. The plasma was separated by centrifugation. The dead fish were measured for length and mass, and the body cavity was opened for gender and maturity determination. The bile was collected by insertion of a needle into the gall bladder and withdrawal into a syringe, and urine (if present) was similarly collected from the urinary bladder. The volumes of bile and urine were noted. For assessment of the percentage of radioactivity in the bloodstream, the total blood volume was assumed to be 1.8% of body weight [16]. All samples were stored on ice for 4–5 h before further processing at Tromsø University and then freezing (−20 °C).

The injection experiment was conducted in accordance with the laws and regulations controlling experiments/procedures in live animals in Norway, i.e. the Animal Welfare Act of December 20th 1974, No 73, chapter VI sections 20–22 and the Regulation on Animal Experimentation of January 15th 1996. The experiment was approved by the Research Institution, id.nr. 124, (Aquaculture Research Station of Tromsø), experiment id. Nr 4753, authorized by The Norwegian Animal Research Authority.

2.2. Processing of bile

The bile from each fish was divided into two halves: one aliquot was frozen (as a back-up), and the other aliquot was extracted. This involved diluting the bile aliquot with deionized water (DW) to 5 mL, and passing it through a solid phase extraction column (SPEC, 820 mg C18, Waters Sep-pak®; www.waters.com) previously primed with 5 mL methanol followed by a 5 mL DW wash. After bile passage, the SPEC was washed with 5 mL DW and eluted with 6 ml of methanol. The methanol was evaporated (45 °C, under a nitrogen stream) and the extracted dry bile sample was frozen (−20 °C). At each stage, 25 μL subsamples were taken for counting (in order to assess the extraction efficiency). In one sample (Fish 3), the majority of the radioactivity passed straight through the SPEC, but was subsequently trapped by passing it through a second SPEC (360 mg C18). Radioactivity was also counted in sub-samples of the water from the bags (1 mL), blood plasma (100 μL) and urine (100 μL). All counts were corrected for volume.

2.3. Thin Layer Chromatography (TLC) of bile extracts

The dry bile extracts were reconstituted in 500 μL of ethanol. TLC of bile extracts was performed on precoated silica gel plates (catalog No. LK6DF; Whatman Labsales; www.whatman.com; but no longer manufactured) for 45 min in an ethyl acetate:ethanol:ammonia solution (45:45:15, v:v:v), which allowed not just free, but also sulfated and glucuronidated steroids to move on the chromatogram. Each sample of bile extract (ca. 20 μL) plus standard(s) was loaded onto a separate lane. TLC lanes were marked and divided into 0.5-cm long intervals, and silica gel from each interval was scraped off the plate. The scrapes were mixed with 500 μL ethanol, 500 μL distilled water and 7 mL scintillation fluid and then scintillation counted for determination of radioactivity.

TLC of steroids produced by solvolysis of bile extracts (see 2.7 below) was carried out with chloroform:ethanol 87:13 (v:v); those treated by chromium trioxide or acetylation with chloroform:ethanol 90:1 (v:v) and those treated by sodium bismuthate or periodic acid with chloroform:ethanol 90:2 (see 2.9 below for detail of microchemistry oxidations and acetylation). Between 5 and 10 μg of standard was spotted onto the plates. The positions of 4-ene steroids were detected by shining a UV lamp on the plate. 5β-androstane-3,11,17-trione was detected by spraying with concentrated sulfuric acid in methanol (10% v/v) and heating the plate for 5 min at 150 °C. All other standard steroids were detected by spraying with a solution of phosphomolydic acid in ethanol (10% w/v) and heating the plate for 5 min at 150 °C. Bands that had been revealed were marked with a pencil and scraped off, as were at least 2 × 0.5 cm lengths of silica gel from either side of the bands. The radioactivity in the silica gel was determined as described above. Identity was assumed when >90% of the radioactivity was associated with the band position of the standard. Standard steroids were purchased from Steraloids (http://steraloids.com). Metabolites of cortisol that were available for purchase were: 11β,17,21-trihydroxypregn-4-ene-3,20-dione (cortisol);