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Development of a validation protocol of enzyme immunoassay kits used for the analysis of steroid hormones in fish plasma

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ABSTRACT

The analysis of steroid hormones 17β-estradiol, testosterone and 11-ketotestosterone in fish plasma provide insight into fish reproductive behavior and development. Typically, steroid hormones are analyzed using gold standard approaches, such as radioimmunoassay and liquid chromatography coupled with tandem mass spectrometry. However, these methods are not always available to small-to-medium research facilities due to their large capital outlay, expensive running costs and ongoing maintenance needs, in addition to requiring a costly radioisotope license and skilled staff to handle and perform the assays. One approach being increasingly used by researchers is commercial enzyme immunoassay (EIA) kits (also known as ELISA kits), analyzed with the use of a UV–vis spectrometer. However, with their increased use, many studies fail to validate their EIA kits or fail to report on the kits sensitivity, accuracy and precision. As such, the aim of this paper is to propose a standardized protocol for reporting EIA kit validation. The proposed protocol was then applied to three fish species in order to demonstrate parallelism, accuracy and precision. Specifically, we tested plasma concentrations of 17β-estradiol for the red and black anemonefish *Amphiprion melanopus*, and 17β-estradiol, testosterone and 11-keto-testosterone for the barramundi *Lates calcarifer* and the common coral trout *Plectropomus leopardus*. Steroid hormones were extracted from fish plasma using a liquid-liquid solvent extraction method with ethyl acetate and n-hexane (50:50 v:v). Following extraction, the plasma dilution curves showed good linearity and parallelism, steroid recoveries were found to be within 90–120% accuracy, and precision variance was < 10% in the majority of assays, for the species and steroids measured. Utilization of the proposed standardized validation protocol of EIA kits will assist scientific, fisheries and aquaculture researchers in obtaining reliable steroid hormone measurements, particularly in the field and in smaller research laboratories.

1. Introduction

The gonadal steroid hormones 17β-estradiol (E2), testosterone (T), and 11-ketotestosterone (11-KT) play important roles in gametogenesis and in regulating reproductive behavior and development of secondary sexual characteristics (Devlin and Nagahama, 2002). In female teleosts (*i.e.*, ray-finned fish), E2 has a well-defined role in vitellogenesis and oocyte maturation (Lubzens et al., 2010; Yaron and Levavi-Sivan, 2011). In male teleosts, the androgens T and 11-KT are associated with spermatogenesis and spermiation (Schulz et al., 2010). Earlier studies on the role of gonadal steroids in teleost reproductive development focused on understanding the endocrine pathways and mechanisms underpinning gametogenesis (Fostier et al., 1983). More recent studies build on this work to examine the role of environmental drivers, such as climate change (Pankhurst and Munday, 2011) and pollution (Kime, 1995; Kroon et al., 2014; Kroon et al., 2015a), in disrupting steroid synthesis and pathways and ultimately reproductive development (Martin-Skilton et al., 2006; Miller et al., 2015). In order to make such assessments, the reliable quantification of gonadal steroid hormones is vital in fish and fisheries research.

To measure plasma steroid hormone concentrations in fish, several standardized methods are available, with radioimmunoassay (RIA) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) based methods considered the gold standard approach (Grebe and Singh, 2011). Briefly, RIA has been the conventional method for measurement of plasma steroid concentrations in fish for decades (Dye et al., 1986; Van der Kraak et al., 1984). However, assay preparation of antisera (*i.e.* blood serum containing antibodies against specific

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antigens) that binds to specific fish steroid hormones is difficult and can be affected by the instability of the radioactive antigen (Kime, 1993). Furthermore, the requirement of specialist license and expensive equipment, combined with accredited monitoring and disposal of radioactive waste make RIA costly, particularly for analyses undertaken in the field and for smaller-to-medium sized research laboratories (Nash et al., 2000).

Methods based on LC-MS/MS are becoming increasingly more common within the academic literature (Penning et al., 2010). LC-MS/MS enables multiple target hormones to be analyzed within a single chromatography trace. However, they too are relatively expensive and are often not available to small-to-medium sized research facilities due to their large capital outlay, expensive running and maintenance costs, and the need for specialized staff trained to analyze the samples and interpret their data (Grebe and Singh, 2011). Furthermore, these methods have been found to lack the sensitivity required to analyze steroid hormone levels due to interferences from secondary metabolites and complex sample matrices (Matuszewski et al., 2003). In addition, it is not uncommon for analyte recoveries to be reported with a large variance (*i.e.*, ± 50%), such as 47.5% and 48.5% recoveries for 10 ng/g and 100 ng/g E2 respectively in *Barbus graellsii* fish homogenates (Jakimska et al., 2013), 129% recovery for 0.31 ng/g 11-KT in *Pimephales promelas* plasma (Hala et al., 2011, and 94.8% and 91.7% recoveries for T and E2 respectively for 5 ng/g spiked mussel samples (Hallmann et al., 2016. Such analyte recoveries and matrix effects are dependent on the MS ionization type, how the samples were extracted and prepared, and the type of biological sample being analyzed. Hormone quantification can be achieved at lower detection limits with more efficient recoveries through optimizing the sampling and extraction protocol for target hormones from different bio-fluids and/or using more specialized extraction procedures and analytical LC columns (Dams et al., 2003). However, this adds to the complexity and difficulty of LC-MS/MS based analyses.

An alternative approach to RIA and LC-MS/MS is the use of Enzyme Immunosorbent Assay (EIA). EIA based methods are widely used to deliver a highly sensitive and reliable quantitative analysis of plasma steroid hormone concentrations in mammalian and non-mammalian vertebrates (Mishra et al., 2007; Nash et al., 2000; Sink et al., 2008; Wust and Hock, 1992). Furthermore, commercial EIA kits have proven to be rapid, easy and relatively cheap to use in quantifying plasma steroid hormone concentrations in fish (Adebiyi et al., 2013; Lorenzi et al., 2012), without producing hazardous by-products such as those formed in RIA. However, a common failing amongst the literature is the lack of guidance on how to validate EIA kits before undertaking steroid measurements, especially if used for the first time on a particular hormone or species. Commercial EIA kits are principally designed to detect hormones in mammalian samples (Kidd et al., 2010), raising the potential for cross-reactivity when quantifying non-mammalian hormonal compounds, especially if not tested by the manufacturer during method development. As such, it is vital that full validation that incorporates all three parameters of parallelism, accuracy and precision becomes the gold standard recommendation for all immunoassay tests (Fischer et al., 2014; Taverniers et al., 2004), especially in collaborative research work. Understanding that the quality of EIA kits vary and that with inadequate kit validation (DuPont et al., 2005) or partial kit validation (*e.g.*, validation of one or two of three parameters), systematic or random errors may be introduced (Andreasson et al., 2015). The researcher thus has a responsibility to ensure rigorous control of assay performance within the scope of the kit used (Bridwell et al., 2010).

To meet this need, a standardized protocol was developed in this study for the use and validation of commercial EIA kits, measuring for parallelism, accuracy and precision (Fig. 1). Firstly, parallelism is undertaken at various sample dilutions in order to ensure target hormones are not saturated and accurate limits of quantification are achieved. Once the optimal sample dilution is obtained from linear curves, sample accuracy and precision is determined *via* analyzing both biological and analytical replicates and spiked samples in order to ascertain kit reproducibility, reliability and recovery from the investigated species and the steroid hormone being investigated. Each stage of the proposed protocol is discussed in detail below, and applied to three fish species in order to demonstrate parallelism, accuracy and precision.

2. Material and methods

2.1. Samples

The developed protocol was applied to three different tropical fish species: the red and black anemonefish *Amphiprion melanopus* Bleeker 1852 (Family Pomacentriadae), the barramundi *Lates calcarifer* (Bloch) (Family Latidae), and the common coral trout *Plectropomus leopardus* (Lacépède) (Family Serranidae), in order to analyze the steroid hormones E2, T and 11KT in fish plasma. The fish selected were based on their extensive use in fisheries research with over a 1000 publications in ISI Web of Science (April 2016), including studies on reproductive endocrinology and ecotoxicology (Frisch et al., 2007; Godwin and Thomas, 1993; Guiguen et al., 1993; Kroon et al., 2014; Kroon et al., 2015a). Species of anemonefish are an important component of the global marine aquarium trade (Wabnitz et al., 2003). Barramundi and coral trout are main predators in tropical estuarine and marine ecosystems (Blaber, 2009; Kingsford, 1992), and support extensive commercial and recreational fisheries (Kailola et al., 1993). All three species are commercially produced in aquaculture operations for ornamental (anemonefish) purposes and food (barramundi, coral trout) (Oliver, 2010; Wabnitz et al., 2003).

Blood samples from all three fish species were taken *via* caudal puncture using a heparinized needle and immediately centrifuged at 10,000*g* for 90 min. Plasma was carefully aspirated from the uppermost layer, transferred in aliquots of 25 µl volumes to 1.5 ml cryogenic polypropylene eppendorf tubes with safe-lock lids and immediately snap-frozen in liquid nitrogen before storing at − 80 °C. The importance of sample stability during long term storage (Shabihkhani et al., 2014) and the detrimental effect of repeated free-thaw cycles (Brey et al., 1994) were recognized to achieve analytical reliability and reproducibility without affecting the concentration of the analyte in plasma. Thus snap-freezing immediately after centrifugation, low temperature storage and only thawing the required aliquots for EIA preparation helped preserve sample integrity.

2.2. Fish collection

2.2.1. Anemonefish

Adult *A. melanopus* breeding pairs were caught from the central Great Barrier Reef between June 2009 and June 2011, and transported to James Cook University, Townsville, Australia (Miller et al., 2015). Blood samples from six females from Miller et al. (2015) were used for this validation study (Table 1), with mean ± SE standard length (SL) of 85 ± 2 mm (range = 78–91 mm) and mean ± SE wet weight of 31 ± 3 g (range = 23–39 g). Due to limited plasma availability at the time of the experiment for *A. melanopus*, only the E2 kit was validated for this species.

2.2.2. Barramundi

Wild *L. calcarifer* were captured in the Tully and Daintree rivers, Far North Queensland, Australia, in 2012 and 2013 (Kroon et al., 2015b). Blood samples from twelve males from Kroon et al. (2015b) were used (Table 1), with mean ± SE total length (TL) of 463 ± 26 mm (range = 377–553 mm) and mean ± SE wet weight of 1133 ± 182 g (range = 600–1825 g).

2.2.3. Coral trout

Wild *P. leopardus*, caught on the northern Great Barrier Reef were purchased from a commercial North Queensland supplier (AustAsia