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Effects of 11-ketotestosterone and temperature on inhibin subunit mRNA levels in the ovary of the shortfinned eel, *Anguilla australis*

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ABSTRACT

Members of the transforming growth factor-b (TGFb) superfamily are important during early oogenesis in mammals. In this study, we tested whether documented effects of 11-ketotestosterone (11KT) on previtellogenic eel ovaries are mediated through affecting the expression of key ovarian TGFb genes. Furthermore, we investigated whether 11KT effects interacted with temperature. Accordingly, three thermal regimes were compared and their interaction with 11KT-mediated actions on expression of TGFb superfamily genes (chiefly inhibin subunits) evaluated in the eel (*Anguilla australis*). Inhibin subunit mRNA levels were also measured in ovarian explants cultured *in vitro* with 11KT and in ovaries from eels collected from the wild. In wild eels, inhibin-bA mRNA levels were higher in early than in previtellogenic eels; inhibin-a expression did not differ between stages, whereas that of inhibin-bB first decreased, then recovered with advanced developmental stage. Temperature was ineffective in modulating any of the end points, at least as long as a Q_{10} adjustment was made to correct for 'metabolic dose'. However, 11KT affected the expression of inhibin-a compared to control fish, while those of inhibin-b subunit genes remained unaffected. In contrast, 11KT dramatically reduced mRNA levels of inhibin-b subunits *in vitro*, but had inconsistent effects on inhibin-a transcript abundance. We conclude that 11KT affects ovarian inhibin subunit gene expression, but effects are not in keeping with the changes seen during early oogenesis in eels from the wild. We further contend that *in vivo* temperature experiments are easily biased and that Q_{10} corrections may be required to identify 'true' temperature effects.

1. Introduction

Environmental factors, especially nutritional status (Izquierdo et al., 2001) and temperature (Van Der Kraak and Pankhurst, 1997), are known to modulate reproduction in fish. There is a myriad of ways in which these factors can exert their effects; for example, temperature could regulate oogenesis by modulating steroidogenesis (reviewed by Van Der Kraak and Pankhurst, 1997) or by affecting base-pairing strength in microRNA repression (Carmel et al., 2012). Furthermore, temperature has been implication in progression of induced vitellogenesis in European eel (*Anguilla anguilla*; Mazzeo et al., 2014). Aside from these environmental factors, the steroid 11KT is known to exert dramatic and wide-ranging effects in anguillids at organismal, cellular and molecular levels; some of these effects are exerted on the ovary during early stages of oocyte growth (c.f., Rohr et al., 2001; Lokman et al., 2007), and our preliminary RNA-Seq data suggest that these effects include modulation of the TGFβ signaling network. Within the TGFβ superfamily, the importance of activins/inhibins and of growth differentiation factor-9 (GDF-9) and its 'sister' bone morphogenetic protein-15 (BMP-15) during early vertebrate oogenesis has been convincingly demonstrated in a range of studies, especially in mammals (*e.g.*, review by Knight et al., 2012).

Activins are homo- or heterodimers of two β-subunits (*e.g.*, Ling et al., 1986), chiefly βA (INHBA) and/or βB (INHBB), whereas inhibin is a heterodimer of one activin subunit and the inhibin-α subunit, INHA (reviewed by Ying, 1988). In the ovary of mammals, inhibin and activin subunit mRNAs (collectively referred to as 'inhibin subunits' in this article) have been localized in the granulosa cells (*e.g.*, Hillier et al., 1989; reviewed in Knight et al., 2012), where they exhibit different patterns of expression during the ovarian cycle. As a generalization (Knight et al., 2012), expression of *Inhba* and *Inhbb* is high during early follicular development, yielding mature activins that are likely to act as autocrine or paracrine regulators (Thompson et al., 2004); later in

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development, as follicles are FSH-responsive, *Inha* expression increases strongly, shifting production to the formation of inhibins (reviewed by Knight et al., 2012). Inhibins can be detected in serum and have a strong inhibitory effect on FSH release (Mason et al., 1985). As in mammals, inhibin subunit mRNAs in fish ovarian follicles localize to somatic cells, for instance, in rainbow trout, *Oncorhynchus mykiss* (Tada et al., 2002; Lankford and Weber, 2010), and in zebrafish, *Danio rerio* (Wang and Ge, 2003; Poon et al., 2009).

Among other TGFβ family members, the effects of GDF-9 and BMP-15 in the mammalian ovary are probably the best defined; these growth factors are secreted as homo- or heterodimeric proteins (Liao et al., 2003; Peng et al., 2013) and are expressed in an oocyte-specific manner from a very early stage to play key roles in promoting early follicular growth (Elvin et al., 2000; Knight and Glister, 2001). In fish, the functions of Gdf-9 and Bmp-15 have not been conclusively shown, but changes in transcript abundance have been documented; for example, *bmp-15* was highly expressed in primary oocytes from sea bass (*Dicentrarchus labrax*) (Halm et al., 2008). In contrast, in zebrafish, *bmp-15* was detected in both oocytes and follicle cells with no significant change in expression during folliculogenesis (Clelland et al., 2006). In the same species, *gdf-9* expression was down-regulated by human chorionic gonadotropin in both ovarian fragments and isolated follicles in a dose- and time-dependent manner (Liu and Ge, 2007).

Information on the TGFβ superfamily system and its functions in the fish ovary is still scanty compared with that in mammals and much of its cellular biology remains poorly understood. Thus, on the back of our preliminary RNA-Seq results, we investigated whether treatment with 11KT *in vivo* and *in vitro* affected the expression of inhibin subunit and of *gdf-9* and *bmp-15* genes in the ovary of the shortfinned eel and whether temperature interacted with the effects of this androgen. We further analyzed the expression of the follicle-stimulating hormone gene (*fshr*) in the ovary and monitored a suite of morphometric indices (organ-somatic indices: eye, gonad, liver, intestine, heart) to serve as positive controls, as these variables have previously been shown to be responsive to androgens (Rohr et al., 2001; Setiawan et al., 2012a,b). A survey of wild eels that were in the pre- (perinucleolar and oil droplet) or early vitellogenic stage was included in the study in order to gain baseline information against which to relate our experimental findings.

2. Materials and methods

2.1. Animals

Wild female shortfinned eels (*Anguilla australis*) in the perinucleolus stage (n = 8), the oil droplet stage (n = 8) or the early vitellogenic stage (n = 8) were captured with fyke nets from Lake Ellesmere, South Island, New Zealand. The fish were euthanized in an overdose of benzocaine (0.3 g/L) very soon after capture and ovarian tissues collected, snap-frozen, and stored at −70 °C until use. Additional details of these same fish, among others pertaining to the lipid physiology of liver and ovary, have been described in detail in a recent report by Damsteegt et al. (in press).

For experimentation, female shortfinned eels in the yellow stage (330–420 g), captured with fyke nets from Lake Waihola, a shallow lake in the South Island of New Zealand, were purchased from commercial eel fishers in late December 2011. Eels were transported to University of Otago fish husbandry facilities and housed in 200 L recirculating tanks equipped with biofilters and temperature control. During acclimation for 1 week, the water temperature was maintained at 15 °C and the photoperiod at 12:12, whereas a salinity of 1/3 SW (11–12 ppt) ensured prevention of fungal outbreaks. Fish were not fed. Manipulations were approved by the University of Otago Animal Ethics Committee, in line with national guidelines defined in the Australian and New Zealand Council for the Care of Animals in Research and Teaching.

2.2. Experimental design

One week after acclimation, experimental eels were anesthetized in Aqui-S (0.2 mL/L) (Day 0) and received intraperitoneal implants of cholesterol and cellulose (19:1; Sherwood et al., 1988) that either did, or did not (controls), contain 0.2 mg 11KT (also see Reid et al., 2013; Lokman et al., 2015). Implants were placed inside the body *via* a small incision (3–5 mm) into the abdominal cavity with a scalpel. Following implantation, eels were assigned to tanks that were pre-set to reach either 10, 15 (the control temperature) or 20 °C within the ensuing 24 h. These temperatures are in the thermo-neutral zone for previtellogenic shortfinned eels and crudely correspond to the range within which feeding activity is observed. Thus, approximate summer temperatures (maximum feeding activity) in Lake Waihola averaged around 23 °C (*e.g.*, Hall and Burns, 2003), whereas the approximate lowest temperature at which feeding in captive shortfins may still occur is around 9 °C (Graynoth and Taylor, 2000). Due to erroneous delivery of some longfinned eels (*Anguilla dieffenbachii*), all eliminated on Day 0, a balanced design could not be used and some variability in sample sizes (3–6 eels/treatment groups) was unavoidable (see Fig. 1). Experiments were run for 21 days, a time interval known to give a measurable response to 11KT treatment on previous occasions (*e.g.*, Forbes, 2013), at each temperature ("fixed-duration" groups). In addition, in order to correct for the decreased and increased metabolic rates at 10 and 20 °C, replicate treatments were set up at these temperatures which were terminally sampled at 33 and 13 days, respectively (see Fig. 1; "fixed metabolic-dose" groups). These experimental durations were calculated on the basis of a $Q_{10} = 2.5$ (*i.e.*, a 2.5-fold change in the rate of biochemical processes for a 10 °C change in temperature), a value that is likely to be applicable for eels within the employed temperature range (c.f., $Q_{10} = 2.45$ in *Anguilla japonica*; Takei and Tsukada, 2001); thus, $Q_5 = \sqrt{Q_{10}} = 1.58$ was used to correct the 21-day sampling regime for metabolic rate (10 °C: 21 × 1.58 = 33 days; 20 °C: 21 / 1.58 = 13 days). Eels remained in excellent condition throughout the experiments and mortality was not observed.

2.3. Terminal sampling

Eels were netted from their tanks and one-by-one placed in an overdose of anesthetic (0.3% benzocaine). Weight and length were recorded and blood was collected and allowed to clot on ice to measure serum steroid levels (Section 2.65). One eye was retrieved and weighed to calculate the eye-somatic index (ESI; eye weight divided by total body weight; we consider this more accurate than measurement of eye diameters, which can suffer from some subjectivity; c.f., Setiawan et al., 2012b). Internal organs (liver, heart, gut, ovary) were subsequently dissected and weighed to similarly calculate somatic indices (organ weight / total body weight). Furthermore, parts of these tissues were frozen on dry ice for quantitative PCR (Sections 2.7 and 2.8) and/or preserved in Bouin's fixative for histological analysis (Section 2.54). All frozen tissues were stored at −70 °C until use. To measure oocyte diameters, freshly dissected ovarian tissue was placed in eel Ringer (see Miura et al., 1991), kept on ice for up to 10 h and subsequently treated with collagenase (2 mg/mL; Sigma Chemical Co., St Louis, MO) for 2 h to separate follicles from their surrounding connective tissue (also see Lokman et al., 2015); separated follicles were then photographed and the five largest oocytes in a field of vision were measured by NIH image software (c.f., Lokman et al., 2007). Steroidogenic abilities of tissues from these fish were previously reported (Reid et al., 2013).

2.4. Effects of 11KT on expression of inhibin subunit genes in vitro

Ovarian tissues from five previtellogenic female shortfinned eels (body weights around 700 g) were cultured in modified Leibovitz L15 medium for 5 days at 18 °C in the presence of 11KT, using a