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Androgens directly stimulate spermatogonial differentiation in juvenile Atlantic salmon (*Salmo salar*)

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ABSTRACT

We studied the effects of androgens on early stages of spermatogenesis along with androgen receptor binding characteristics and the expression of selected testicular and pituitary genes. To this end, immature Atlantic salmon postsmolts received testosterone (T), adrenosterone (OA, which is converted in vivo into 11-ketotestosterone, 11-KT) or a combination of the two androgens (T + OA). Treatment with OA and T elevated the plasma levels of 11-KT and T, respectively, and co-injection of OA with T lead to high 11-KT levels but prevented plasma T levels to reach the levels observed after injecting T alone. Clear stimulatory effects were recorded as regards pituitary *lhb* and *gnrhr4* transcript levels in fish receiving T, and to a lesser extent in fish receiving OA (but for the *lhb* transcript only). The two androgen receptors (Ara1 and Ara2) we cloned bound T and 11-KT and responded to these androgens in a similar way. Both androgens down-regulated testicular *amh* and increased *igf3* transcript levels after 1 week of treatment, but effects on growth factor gene expression required sustained androgen stimulation and faded out in the groups with the decreasing T plasma levels. In fish exhibiting a sustained elevation of 11-KT plasma levels (OA and T + OA groups) for 2 weeks, the number of differentiating spermatogonia had increased while the number of undifferentiated spermatogonia decreased. Previous work showed that circulating gonadotropin levels did not increase following androgen treatments of gonad-intact immature male salmonids. Taken together, androgen treatment of immature males modulated testicular growth factor expression that, when sustained for 2 weeks, stimulated differentiation, but not self-renewal, of undifferentiated type A spermatogonia.

1. Introduction

Pubertal gonad maturation in vertebrates usually requires stimulation by the pituitary gonadotropins luteinizing hormone (Lh) and follicle-stimulating hormone (Fsh). In salmonid fish, however, Fsh is the only gonadotropin available in the blood during the onset of puberty and increased pituitary and plasma Fsh levels were associated with elevated androgen levels and spermatogonial proliferation and differentiation activity (Melo et al., 2014; Maugars and Schmitz, 2008; Campbell et al., 2003; Gomez et al., 1999). The main androgen produced by fish testes is 11-ketotestosterone (11-KT) (Borg, 1994) but significant concentrations also of testosterone (T) are found in the blood of salmonid species (e.g. Freeman et al., 1983; Liley et al., 1986; Scott and Sumpter, 1989), and both T and 11-KT are involved in the feedback control of reproduction in salmonids (e.g. Antonopoulou et al., 1999; Goos et al.,

Abbreviations: 11-KT, 11-ketotestosterone; Amh, anti-Müllerian hormone; Fsh, follicle stimulating hormone; gnrhr4, gonadotropin-releasing hormone receptor 4; Igf3, insulin-like growth factor 3; Lh, luteinizing hormone; OA, adrenosterone; T, testosterone.

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1986). Fsh is a potent steroidogenic gonadotropin in fish (Chauvigné et al., 2012; García-López et al., 2010, 2009; Planas and Swanson, 1995) and in prepubertal Japanese eel (*Anguilla japonica*), Fsh-stimulated androgen production mediated the stimulatory effect of Fsh on spermatogenesis (Ohta et al., 2007). However, studies in trout (*Oncorhynchus mykiss*; Sambroni et al., 2013a) showed that Fsh can also directly change Sertoli cell transcript levels, including transcripts encoding growth factors that modulate germ cell differentiation, such as *amh* (Skaar et al., 2011; Miura et al., 2002). Moreover, androgens can change testicular mRNA levels coding for proteins potentially relevant for the onset of spermatogenesis (Rolland et al., 2013). Thus, Fsh can directly modulate Sertoli cell functions, or first stimulate Leydig cell androgen production that then modulate spermatogenesis via androgen receptor expressing testicular somatic cells.

The decreases in testicular *amh* mRNA levels during gonadotropin-induced (Miura et al., 2002) or natural puberty (Maugars and Schmitz, 2008) as well as the fact that Amh blocked androgen-induced spermatogenesis in prepubertal eel (Miura et al., 2002) are interesting to note. In adult zebrafish (*Danio rerio*), Amh inhibited the differentiation of type A spermatogonia, and moreover inhibited Fsh-stimulated steroidogenesis (Skaar et al., 2011). Members of the insulin-like growth factor (Igf) family, on the other hand, stimulated germ cell proliferation in trout (Loir, 1999), or were required as permissive factor for androgen-stimulated spermatogenesis in eel (Nader et al., 1999). Interestingly, a new member of this family, Igf3, has been described recently (Wang et al., 2008). This gene is predominantly expressed in gonadal tissue and its expression is up-regulated by Fsh in zebrafish (de Waal, 2009; Baudiffier et al., 2012) and in rainbow trout testis (Sambroni et al., 2013a). However, a quantitative evaluation of androgen-induced germ cell development in combination with quantifying testicular mRNA levels of *amh* and *ifg3* has not been reported so far.

Thus, in order to evaluate the effects of sex steroids on the initiation of pubertal spermatogenesis, we treated immature male fish with two different androgens. Blood (11-KT and T levels) and testis samples (*amh* and *igf3* transcript levels; quantitative evaluation of germ cell development) were collected for analyses after 1 and 2 weeks. Since sex steroids also modulate pituitary gene expression, we measured *fshb*, *lhb* and *gnrhr4* mRNA levels. Moreover, 11-KT and T had in part similar, in part distinct effects on testicular gene expression patterns in rainbow trout (Rolland et al., 2013). In order to evaluate the biological activities of these androgens via the androgen receptor (Ar), we cloned two androgen receptor subtypes and studied their ligand binding characteristics.

2. Material and methods

2.1. Experimental design

The experiments took place at Matre Research facility (61°N), which is part of the Institute of Marine Research, Bergen (Norway). In a pilot study, different doses of testosterone (Sigma, St. Louis, USA) and adrenosterone (OA; Sigma, St. Louis, USA) were tested on immature Atlantic salmon post-smolts (length 23.6 ± 0.2 cm, body weight 155.5 ± 3.2 grams) in seawater (35 ppt salinity) at a water temperature of 16 °C. To that end, androgens were dissolved in ethanol and then added to 1 part vegetable oil and 1 part shortening (both from Crisco; J.M. Smucker Company, USA), according to Specker et al. (1994). The steroid solution was kept liquid at 25 °C and injected into the body cavity between the pelvic and the anal fin. Only after injecting 25 μg T/g body weight, the highest of three doses tested in the pilot study, increased T plasma levels were recorded after 4 days but had returned to basal levels after 8 days (Fig. 1A). After injection of all doses of OA (1, 5, and 25 μg/g body weight), similarly elevated 11-KT plasma levels were recorded on day 4. Interestingly, increasing doses resulted in an extension of the time period during which elevated 11-KT plasma levels were found but not in higher plasma levels (Fig. 1B). The pilot study also showed that although equal doses were administered, T plasma levels were at maximum only half as high as 11-KT levels. Therefore, for the main experiment, we tripled the dose of T compared to the one of OA. In a previous study on African catfish (*Clarias gariepinus*), 3-fold higher doses of T than of the 11-oxygenated androgen had to be administered to achieve similar circulating levels (Schulz et al., 2008). Hence, 160 Atlantic salmon postsmolts (length 22.3 ± 1.3 cm, body weight 120.6 ± 22.3 g) were injected with either a fat solution containing no steroid (C), 75 μg T/g of body weight (T75), 25 μg OA/g of body weight (OA25), or a combination of the two androgens (T75 + OA25), establishing four groups with 40 fish each. OA is converted in Atlantic salmon in vivo into 11-KT (Antonopoulou et al., 1999; Borg et al., 1998; Mayer et al., 1990), the main androgen in fish (Borg, 1994). The fish were maintained in four 500 L seawater square tanks under continuous light conditions (at 35 ppt salinity and a water temperature of 12 °C) and fed with commercial feed (Spirit 75, Skretting AS, Stavanger, Norway) distributed by automatic feeders to satiation throughout the whole experimental period.

The experiments have been approved by the local responsible laboratory animal science specialist under the surveillance of the Norwegian Animal Research Authority (NARA) and registered by NARA.

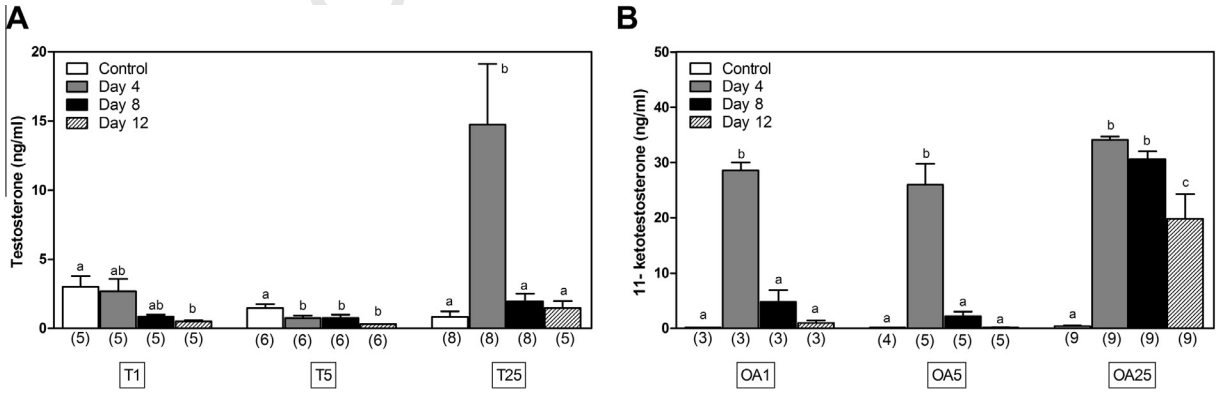

Fig. 1. Plasma androgen levels of male Atlantic salmon treated with different androgen doses in a dose-finding pilot experiment. Testosterone (A) and 11-Ketotestosterone (B) plasma levels after 4, 8 and 12 days of androgen injection. Bars show mean values ± SEM for the three different concentrations used (1, 5, or 25 μg/g body weight): T(1), T(5), T(25) and OA(1), OA(5), OA(25). Different lower case letters denote means that are significantly different time-wise ($P < 0.05$; one-way ANOVA followed by SNK test). The number of males per group is indicated between brackets under the respective bars.

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