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Extra-gonadal steroids modulate non-breeding territorial aggression in weakly electric fish

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ABSTRACT

The neuroendocrine control of intraspecific aggression is a matter of current debate. Although aggression in a reproductive context has been associated with high levels of circulating androgens in a broad range of species, it has also been shown to occur during the non-breeding season when gonads are regressed and plasma steroid hormone levels are low. In mammals and birds the aromatization of androgens into estrogens plays a key role in the regulation of aggression in both the breeding and non-breeding seasons. This is the first study in a teleost fish to explore the role of steroids in the modulation of non-breeding aggression. *Gymnotus omarorum* is a highly aggressive teleost fish that exhibits aggression all year-round. We analyzed male–male non-breeding agonistic behavior, compared circulating 11-Ketotestosterone (11-KT) levels between dominants and isolated males, assessed the regulatory role of aromatization of androgens into estrogens, and evaluated the gonads as a source of these sex steroids. We found that high levels of aggression occurred in the non-breeding season despite low plasma 11-KT levels, and that there was no difference in 11-KT levels between dominant and isolated males. We demonstrated that acute aromatase inhibition decreased aggression, distorted contest dynamics, and affected expected outcome. We also found that castrated individuals displayed aggressive behavior indistinguishable from non-castrated males. Our results show, for the first time in teleost fish, that territorial aggression of *G. omarorum* during the non-breeding season depends on a non-gonadal estrogenic pathway.

Introduction

Virtually all organisms display intraspecific aggression in their agonistic social behaviors, and its neuroendocrine control is a matter of current debate. It is generally accepted that gonadal testosterone (T) stimulates male–male aggression in reproductive contexts in a broad range of vertebrate species. Castration–hormone replacement experiments have set forth undisputable evidence strongly supporting the role of gonadal testosterone (Balthazart, 1983; Nelson, 2005; Wingfield et al., 1990; Wingfield and Hahn, 1994). Nevertheless, there are other factors that indicate that regulation of aggression is more complex. In this sense, the aromatization of androgens into estrogens plays a key role in the promotion of aggression during the breeding season. Schlinger and Callard (1990) performed the first experiments that showed that while high levels of circulating T and estradiol (E2) promoted aggressive behavior in the quail *Coturnix coturnix japonica*, aggression was greatly reduced by aromatase inhibitors and estrogen receptor (ER) blockers but not by androgen receptor antagonists. Furthermore, nuclear ERs have been shown to be key in breeding male–male aggression in mammals: ERα knockout mice show decreased aggression (Ogawa et al., 1997; Scordalakes and Rissman, 2003) and the number of ERα positive cells in several brain regions is positively correlated with aggression (Trainor et al., 2006).

Interestingly, aggression is dissociated from the breeding season in many species, and robust territorial aggression can occur when gonads are regressed, circulating T levels are low, and even after castration in birds (Gwinner et al., 1994; Logan and Wingfield, 1990; Soma and Wingfield, 1999; Wingfield, 1994; Wingfield et al., 1997), mammals (Caldwell et al., 1984; Demas et al., 2007; Jasnow et al., 2000) and reptiles (Moore and Marler, 1987). As in the breeding season, estrogens are involved in regulating non-breeding territorial aggression in birds, although their source is most probably not gonadal (Hau et al., 2000; Schlinger et al., 1992; Soma and Wingfield, 1999; Soma et al., 1999, 2000a,b). It has been shown that androgens and estrogens can be synthesized locally in the brain either from extragonadal precursors, such as adrenal dehydroepiandrosterone (DHEA) (Demas et al., 2007; Soma and Wingfield, 2001; Soma et al., 2008) or possibly de novo (Tsutsui and Yamazaki, 1995; Tsutsui et al., 2003). Aromatase activity changes seasonally in a brain region-specific manner and correlates with changes in aggression (Soma et al., 2003). Aggressive interactions can also produce changes of E2 levels in specific brain areas (Charlier et al., 2011). Estrogens have been shown to be potent neurosteroids with

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rapid effects (Balthazart et al., 2006; Cornil et al., 2006; Pradhan et al., 2008, 2010; Remage-Healey et al., 2010; Taziaux et al., 2007; Woolley, 2007) and can rapidly increase aggressive behavior and intracellular signaling in brain regions associated with social behavior with time courses that differ across seasons (Heimovics et al., 2012; Trainor et al., 2007, 2008). Signaling pathways shift from predominately genomic mechanisms (effects in >30 min) in the breeding season, to mainly non-genomic ones (<30 min) in the non-breeding season (Heimovics et al., 2012, 2015; Trainor et al., 2007, 2008). Together, these results call for the exploration of other taxa to understand if estrogenic regulation of non-breeding aggression is a general feature of vertebrates.

In teleost fish, as in other taxa, levels of aggression in the breeding season are positively correlated to circulating androgens and E2 (reviewed in Borg, 1994; Huffman et al., 2012; O'Connell et al., 2013), exogenous administration of androgens (reviewed in Gonçalves and Oliveira, 2010), levels of sex steroid brain receptor transcripts (Maruska and Fernald, 2010; Morandini et al., 2014; Oliveira et al., 1996; Parikh et al., 2006; Ramallo et al., 2014) and brain expression of aromatase (Huffman et al., 2013). Furthermore, breeding aggression decreases with castration (Francis et al., 1992), blockage of androgenic pathways (Sessa et al., 2013), and inhibition of aromatase (Huffman et al., 2013). Studies of non-breeding territorial aggression in teleosts are extremely scarce (Batista et al., 2012; Vullioud et al., 2013) and the underlying neuroendocrine mechanisms remain unknown.

The weakly electric fish *Gymnotus omarorum*, a well-established neuroethological model (reviewed in Caputi et al., 2005; Lorenzo et al., 2006; Silva et al., 2002), shows inter- and intrasexual non-breeding territorial aggression (Batista et al., 2012; Zubizarreta et al., 2012, 2015) modulated by neuropeptides (Silva et al., 2013) and social context and monoamines (Zubizarreta et al., 2012, 2015). The electric organ discharge (EOD) plays a key role in communication, signaling submission during aggressive encounters and subordinate status once rank is determined (Batista et al., 2012; Silva et al., 2013).

G. omarorum offers a unique opportunity to examine the neuroendocrine regulation of non-breeding aggression and identify common strategies that may have emerged across vertebrates. In this study we describe two crucial results that establish *G. omarorum* as a valuable model: a) we show the importance of sex steroids in the rapid modulation of teleost male aggression in the non-breeding season, as the aromatization of androgens is critical for the full expression of the behavior, and b) we confirm that the testes are not the source of these sex steroids, as castrated males display aggressive behavior indistinguishable from non-castrated males.

Materials and methods

Animals

We used 54 adult male *G. omarorum* (Richer-de-Forges et al., 2009) (body-length 15–25 cm and body-weight 8.9–44.5 g). As this species is sexually monomorphic, sex was identified by gonadal inspection. All experiments were carried out during the non-breeding season (May–August) (Silva et al., 2003).

Fish were collected in the "Laguna del Sauce" (34°51′S, 55°07′W), Maldonado, Uruguay using an electrical detector as previously described by (Silva et al., 2003). To prevent the experience of recent agonistic interactions (Hsu et al., 2006), animals were housed in individual compartments within large outdoor tanks (500 L). These tanks were under natural photoperiod (from LD 10:14 to LD 11:13), temperature (10.41 ± 3.48 °C), and conductivity (<200 μS/cm) (Silva et al., 2003) and were covered by aquatic plants. Each compartment was provided with one shelter for the fish. Fish were fed ad libitum with *Tubifex tubifex*.

All experiments were performed according to the regulations for the use of animals in research and the experimental protocol was approved by the institutional Ethical Committee of Instituto de Investigaciones Biológicas Clemente Estable (IIBCE) (Resolution 10/05/2012).

Behavior

We observed the agonistic behavior of *G. omarorum* in dyadic male–male encounters and tested the effect of aromatase blocking and of castration during the non-breeding season. Fifty four males (identified by surgical gonadal inspection) were randomly assigned to a dyad respecting a 5–20% weight difference between contenders. As contest outcome in *G. omarorum* depends on fish size asymmetries (Batista et al., 2012), we were able to predict the expected dominant (large fish) and subordinate (small fish). The dyads were randomly assigned to one of the following experimental groups: fadrozole-treated dyads (FAD dyads, n = 8), saline-treated dyads (saline dyads, n = 8), gonadectomized dyads (GDX dyads, n = 5) and sham-gonadectomized dyads (SHAM dyads, n = 6). We carried out dyadic contests between individuals of these experimental groups, evaluated agonistic behavior (contest outcome, dynamics, aggression, and submission levels), and compared it across experimental groups: FAD dyads versus saline dyads, and GDX dyads versus SHAM dyads. This experimental design ensured that: a) all experimental groups were composed of fish spanning the same size range; b) fish were randomly assigned to a dyad in which the weight difference between contenders allowed us to predict the expected outcome; and c) each fish was used only once to avoid the effects of previous experience.

Fish dyads were placed in a behavioral setup (as described in Silva et al., 2007) that allowed simultaneous video and electric recordings, control of photoperiod, water temperature, conductivity, and pH. The setup consists of 4 experimental tanks (55 × 40 × 25 cm) divided in half by a removable glass gate. All experiments were performed at night, in darkness, with infrared LED illumination (Kingbright L-53F3BT; 940 nm) located above the tanks. Experiments were filmed with an infrared-sensitive video camera (SONY CCDIris, Montevideo, Uruguay) through the glass bottom of the tank. The electric signals of freely moving fish were detected by two pairs of fixed copper wire electrodes connected to two high-input impedance (1 MΩ) amplifiers (FLA-01; Cygnus Technologies Inc., Delaware Water Gap, PA, USA). Images and electric signals were captured by a video card (Pinnacle Systems, PCTV-HD pro stick) and stored in the computer for further analysis.

For agonistic encounters, we used a plain arena (without food or shelter) and simultaneously placed each contender in one of the equally sized compartments 2 h prior to the experiment, thus providing equal resources (territory and residency) to each individual (Batista et al., 2012). The glass gate was raised 10 min after sunset, and fish were separated 10 min after conflict resolution. Dominant and subordinate fish were then removed from the tank. In experiments testing the effect of gonadectomy on agonistic behavior, both GDX and SHAM fish were immediately anesthetized with 2-phenoxyethanol (Sigma, 0.075%), bled to obtain samples for hormone measurements and afterwards euthanized with 2-phenoxyethanol (0.1%).

Basic surgery: surgical gonadal inspection

All fish were subjected to a basic surgery for gonadal sex identification. Fish were individually housed in indoor tanks of 20 × 30 × 20 cm at least 24 h before surgery for acclimation to the new environment. Individuals were anesthetized with 2-phenoxyethanol 0.075%, artificially ventilated, and a small lateral incision was performed for visual identification of gonadal sex (under a stereo microscope). After surgical suture, fish were treated with topic silver nitrate solution, 1%; and amoxicilline, 0.0125%, in the water. After full cicatrization and stabilization of body weight, fish were returned to their outdoor housing.