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TITLE: Experimentally-induced variation in neuroendocrine processes affects male reproductive behavior, sperm characteristics, and social interactions.

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ABSTRACT: While extensive research has focused on how social interactions evolve, the fitness consequences of the neuroendocrine mechanisms underlying these interactions have rarely been documented, especially in the wild. Here, we measure how the neuroendocrine mechanisms underlying male behavior affecting mating success and sperm competition in the ocellated wrasse (Symphodus ocellatus). In this species, males exhibit three alternative reproductive types. 'Nesting males' provide parental care, defend territories, and form cooperative associations with unrelated 'satellites', who cheat by sneaking fertilizations but help by reducing sperm competition from 'sneakers' who do not cooperate or provide care. To measure the fitness consequences of the mechanisms underlying these social interactions, we used "phenotypic engineering" that involved administering an androgen receptor antagonist (flutamide) to wild, free-living fish. Nesting males treated with flutamide shifted their aggression from sneakers to satellite males and experienced decreased submissiveness by sneaker males (which correlated with decreased nesting male mating success). The preoptic area (POA), a region controlling male reproductive behaviors, exhibited dramatic down-regulation of androgen receptor (AR) and vasotocin 1a receptor (V1aR) mRNA following experimental manipulation of androgen signaling. We did not find a direct effect of the manipulation on male mating success, paternity or larval production. However, variation in neuroendocrine mechanisms generated by the experimental manipulation was significantly correlated with changes in behavior and mating success: V1aR expression was negatively correlated with satellite-directed aggression and expression of its ligand arginine vasotocin (AVT) was positively correlated with courtship and mating success, thus revealing the potential for sexual selection on these mechanisms.

Introduction

Extensive research has documented selection on and arising from the amazing diversity of reproductive and social behaviors that exist in nature. The neural and hormonal mechanisms underlying variation in reproductive and social behaviors have also been studied extensively (e.g. Adkin-Regans 2005; O'Connell & Hofmann 2012; Kalueff *et al.* 2014; Rodgers *et al*., 2013). In contrast, a relatively small number of studies have linked mechanism, behavior and fitness by examining how natural or experimentally-induced variation in circulating hormones is associated with variation in behavior and fitness (e.g. Rohwer & Rohwer 1978; Ketterson et al. 1992; Aubin-Horth *et al.* 2007; Veiga & Polo 2008; Sinervo *et al.* 2000; Mills *et al.* 2007, 2008, 2009; McGlothlin *et al*. 2007, 2008, 2010). These existing studies have demonstrated the potential for selection to shape the hormonal mechanisms underlying behavior (e.g. reviewed in Ketterson *et al.* 1996; Ketterson & Van Nolan 1999; McGlothlin & Ketterson 2008). The fitness consequences of variation in the neural and hormonal basis of social interactions are very challenging to measure due to the difficulty of performing mechanistic experiments on wild populations that still allow social interactions and reproduction to occur in otherwise natural conditions. Fully understanding how these mechanisms arose, however, requires measuring the fitness consequences of variation in the mechanisms underlying these behaviors in the social and ecological context in which they evolved (Linnen & Hoekstra 2009). In addition, the few existing studies that focused on the relationship between hormones, behavior and fitness have not simultaneously measured neural gene expression. Though genetic polymorphisms have been associated with variation in mating tactics (e.g. Lemperte *et al.* 2010), we are not aware of any studies that simultaneously measured the fitness consequences of variation in the neural and hormonal mechanisms underlying social interactions under natural conditions in the wild. To address this gap in our knowledge, we experimentally manipulated neuroendocrine mechanisms known to affect male social behavior in free-living vertebrate fishes and measured how this experimentally-induced variation affected neural gene expression, individual behavior, social interactions,

and male reproductive success under natural conditions in the wild. We find that experimentally-induced variation in the underlying neuroendocrine processes is associated with changes in male behavior and social interactions.

The ocellated wrasse (*Symphodus ocellatus*) is an emerging model for connecting variation in physiology to variation in social behavior and reproductive success in a freely-behaving, wild animal (Stiver *et al.* 2014; Nugent *et al.* 2016; Dean *et al.* 2017). In *S. ocellatus*, three discrete male alternative types exist (Warner & Lejeune 1985; Taborsky *et al.* 1987, Alonzo *et al.* 2000). Large, colorful nesting males are socially dominant, build and defend nests, court females, and engage in paternal care (e.g. fanning the developed eggs and tending the nest). Small, parasitically breeding sneaker males opportunistically release sperm (spawn) in nesting males' nests without engaging in courtship or paternal care (Warner & Lejeune 1985; Taborsky *et al.* 1987; Alonzo 2004). The nesting male tolerates an intermediate morph, the satellite male, near his nest as satellites assist with courtship and nest defense, although they do not engage in parental care (Taborsky *et al.* 1987; Stiver & Alonzo 2013). The satellite capitalizes on this tolerance by sneak spawning when the nesting male is distracted (Stiver & Alonzo 2013). During their breeding season, nesting males guard, tend, and spawn in their nests and are highly aggressive toward conspecific males and heterospecific egg predators (Lejeune 1985). In the ocellated wrasse, sperm competition plays a critical role in determining male reproductive success. Sneak spawning is rampant at active nests, with cuckoldry occurring at 100% of nests (Alonzo & Heckman 2010). Sneaker males pose a large threat to the reproductive success of nesting males since they release more sperm per spawn compared to the other male morphs, and the number of sneakers at a nest directly correlates with the intensity of sperm competition (Alonzo & Warner 2000). However, since sneakers and satellite males do not engage in paternal care, their reproductive success relies on parental care by the nesting male. The behavior of one individual can therefore have marked fitness consequences for the entire social group breeding at a nesting site.

Recent research on the ocellated wrasse has identified mechanisms underlying some of this striking variation in behavior under natural conditions in the wild. In general, neuroendocrine signaling mediates social and sexual behaviors across vertebrate taxa (Goodson 2005). Studying neuroendocrine systems is of particular interest in animals with alternative reproductive tactics because differences in hormone signaling likely contribute to variation in reproductive tactics and success (Knapp 2003). High levels of testicular androgens have been linked to dominance, aggression, and male reproductive behaviors in several teleost fish species (Oliveira *et al.* 2001a; Parikh *et al.* 2006; Desjardins *et al.* 2008; Schradin *et al.* 2009; Taves *et al.* 2009; O'Connell & Hofmann 2011a; Rodgers *et al.* 2013; Pradhan *et al.* 2014). In the ocellated wrasse, 11-ketotestosterone (11-KT) is the primary androgen regulating male social and reproductive behaviors; circulating levels of this potent gonadal hormone are elevated in nesting males compared to sneaker and satellite males (Stiver *et al.* 2014; Nugent *et al.* 2016).

Gonadal steroid hormones and their nuclear receptors – which can act as transcription factors or via noncanonical signaling pathways– are also well-known regulators of gene expression and are therefore powerful determinants of an animal's physiological and behavioral phenotype (Evans 1988; Beato 1993). Hormone concentrating regions of the brain (areas with high densities of steroid hormone receptors) are critical for reproductive and social behavior (Goodson 2005; O'Connell & Hofmann 2011b, 2012). The preoptic area (POA) is widely accepted as a critical node controlling male sexual behavior across taxa (Heimer & Larsson 1967; Arendash & Gorski 1983; Koyama *et al.* 1984). Neuroendocrine signaling in the ventromedial hypothalamus (VMH, sometimes called the anterior tuberal nucleus in teleosts), although typically associated with female reproductive behavior in mammals (Malsbury *et al.* 1977; Frankfurt *et al.* 1990; Mani *et al.* 1994; Bale *et al.* 2001; Musatov *et al.* 2006), is also critical for male-typical behaviors and mediation of aggression toward conspecifics in territorial animals (Olivier 1977; Nelson & Chiavegatto 2001; Lin *et al.* 2011). Androgen receptor (AR) expression is high in both the preoptic area (POA) and

ventromedial hypothalamus (VMH) of teleost fish compared to other brain regions (Harbott *et al.* 2007; Munchrath & Hofmann 2010), and high expression of brain androgen receptor (AR) has been linked to social dominance (Burmeister *et al.* 2007). Androgens can be aromatized in the brain to estradiol (Naftolin 1994), which binds to estrogen receptors α and β (ERα, ERβ), which can also mediate social behaviors in teleosts (Saaristo *et al.* 2010; Huffman, O'Connell and Hofmann 2011).

In addition to gonadal steroids, peptide hormones, such as arginine vasotocin (AVT; homologue to the mammalian vasopressin (AVP)) and its primary receptor, the vasotocin 1a receptor (V1aR), have also been the focus of studies on social and reproductive behaviors in fish and other taxa (Greenwood *et al.* 2008; Oldfield & Hofmann 2011; Huffman *et al.* 2015). The AVT/AVP system has been implicated in a wide range of behaviors critical to an animal's survival and fitness, such as territorial aggression, courtship, and parental care (reviewed in (Insel & Young 2000; Goodson & Bass 2001; Donaldson & Young 2008; Insel 2010; Oldfield et al. 2015). Importantly, the V1aR is highly sensitive to regulation by circulating androgens, which increase its expression, specifically in the preoptic area (Young *et al.* 2000). To understand the mechanisms underlying these behaviors, it is therefore essential to not only study circulating hormones but also document how they affect gene expression in these regions of the brain.

We therefore investigated how variation in neuroendocrine function among male ocellated wrasses affects neural gene expression patterns associated with social behavior, social interactions and reproductive success (under otherwise natural conditions in the wild) by experimentally manipulating androgen receptor signaling in nesting males. To accurately measure the fitness consequences of any changes in neural gene expression and associated changes in social behavior and interactions arising from this manipulation, it was critical to perform this study on free-living organisms experiencing their natural social and ecological conditions. Here we show that dominant nesting males can be readily captured in

the wild, pharmacologically manipulated, and returned to their nest where they display little to no detectible disturbance in their natural behaviors. This allows mechanistic studies to be completed in the wild, providing powerful links between physiology, behavior, and reproductive success. Moreover, we measured male mating and fertilization success by first directly observing mating success before and after the experimental manipulation and second collecting the larvae from each male's nest for genetic paternity analyses (Alonzo & Heckman 2010; Stiver & Alonzo 2013). We found that manipulating androgen signaling in nesting males altered sperm characteristics, neural gene expression and circulating hormones. This experimentally-induced variation in androgen signaling also altered nesting male behavior and social interactions with the sneaker and satellite males with which they are competing for mates and fertilizations.

MATERIALS AND METHODS

To document the social and fitness consequences of variation in the neuroendocrine mechanisms underlying male social behavior and interactions during reproduction, we manipulated AR signaling in twenty (n=10 flutamide, n=10 vehicle) free-living nesting males and measured how this affected male reproductive physiology, behavior, social interactions, mating success and reproductive success under natural conditions in the wild. We also conducted a laboratory study on 17 nesting males (n=10 flutamide, n=7 vehicle) to examine how the manipulation affected male gonad size, sperm production and sperm characteristics. We used this phenotypic engineering (*sensu* Ketterson *et al.* 1996) approach to measure the overall treatment effects of manipulating androgen signaling and to generate greater phenotypic variation in the neuroendocrine mechanisms underlying male social behavior. There are two ways of drawing inference based on the results of such phenotypic engineering. First, we can ask how the treatment (flutamide or vehicle) affects whether, in what direction and how much each variable of interest changes before versus after the manipulation. Second, we can think of the manipulation as generating greater phenotypic variation than normally observed which allows us to ask whether and how this resulting

variation is associated with variation in behavior, mating and reproductive success. We use both approaches in this stud and report both kinds of analyses in the results below.

Animals

Field studies were conducted between mid-May and mid-June in 2013 at the University of Liège Marine Laboratory (La Station de Recherches Sous-Marine et Océanographique, STARESO), near Calvi, Corsica, France. The breeding season for *S. ocellatus* lasts approximately two months (May, June and sometimes into July in certain regions of the Mediterranean Sea). Fish were observed and caught on SCUBA at nest depths ranging from 2 to 12 meters. Only nesting males from actively spawning nests with a satellite and at least two sneakers were used, as the goal of the study was to examine how the experimental manipulation affected social interactions among alternative male types, mating success, and reproduction. All procedures were conducted with approval by Yale University's Institutional Animal Care and Use Committee.

Flutamide Injection

Following an initial baseline behavioral observation (details below), twenty nesting males were caught and transported to the field laboratory where they were measured, weighed, fin clipped for genotyping and given a 20 μl intraperitoneal (IP) injection of either flutamide (Sigma; 2.5 μg/g body weight; based on Oliveira *et al.* 2009; Dang *et al.* 2011; Bayley *et al.* 2002; Soffker & Tyler 2012) or vehicle (sesame oil; n=10 nesting males per treatment). Animals were given unique identifying marks with superficial injections of visible implant elastomer (Northwest Marine Technology, Inc.) under their ventral or dorsal skin. While experimental nesting males were injected, their nests were protected with a mesh hand net to prevent predation or take-over by nearby nesting males. Males were returned to their nests within 30 minutes of injection. An additional 17 nesting males were caught from

actively spawning nests, given an injection (n=10 flutamide, n=7 vehicle) and held in the lab for further study (for further details see the section on “Sperm Characterization” below).

Behavioral Observation and Analysis

An initial 5-minute observation was video recorded prior to nesting male removal and injection to provide a baseline for comparison. Additional 5-minute video observations were recorded for three consecutive days following injection. Behavioral recording and scoring was completed by a researcher blind to treatment group. Behavior of all males at a given nest was recorded as described in Alonzo & Warner 2000. In addition, we recorded the number of sneakers and females present at the nest and the nesting male’s proximity to the nest. Behavior scores on the 3 days post-injection were averaged to control for variation over time and to allow us to compare behavior before versus after the experimental manipulation.

Tissue Collection

On the final day of behavioral observations, the twenty nesting males included in this study were caught a second time and briefly placed in seawater containing MS-222 (Sigma) until motor function was no longer observed. Blood was immediately collected from the dorsal aorta with a heparinized 26-gauge butterfly needle (Becton Dickson) into heparinized tubes. Collected blood was placed on ice, and then spun for 10 minutes at 6000 rpm to separate plasma. Plasma was stored at -20°C at the field station, transported to The University of Texas at Austin then stored at -80°C until 11-ketotestosterone was measured. Immediately following blood collection, nesting males were rapidly decapitated, their brains were removed and placed in RNAlater (Ambion) and stored at -20°C. Gonads were removed and weighed to quantify the male’s gonadosomatic index (GSI, i.e. gonad mass/total body mass). Brains were embedded in TissueTek O.C.T. compound (Sakura Finetek) and sectioned at 300 µm on a cryostat. The POA and VMH were bilaterally microdissected from the appropriate brain section using a 300 µm diameter tissue punch (Fine Science Tools).

Tissue punches were submersed in 180 µl of RNA/DNA shield (Zymo Research) and stored at -80°C until processed.

11 – Ketotestosterone Quantification

Circulating levels of free 11-ketotestosterone were quantified using ELISA (Cayman Chemicals) on plasma samples diluted 1:30 in assay buffer from the ELISA kit according the manufacturer's protocol and as previously described (Kidd *et al.* 2010). All standards and experimental samples were assayed in duplicate by an experimenter blind to sample treatment group. Optical density was measured using a Spectramax M3 plate reader (Molecular Devices) and samples were compared to a standard curve to quantify circulating 11-ketotestosterone. Differences in 11-kt between flutamide and vehicle-injected nesting males were assessed by two-tailed t-test in R (R Development Core Team 2011) with $\alpha < 0.05$. We focus here on 11-kt and did not measure testosterone in this study, as testosterone does not appear to play a major role in nesting male specific behaviors (Stiver *et al.* 2014; Nugent *et al.* 2016), consistent with 11-kt as the dominant androgen in teleosts (Kindler *et al.* 1989; Rodgers *et al.* 2006). Ocellated wrasses are relatively small (e.g. nesting males are ~7-9 cm standard length). It was therefore not possible to take daily blood samples during the study (e.g. on a daily basis following the initial injection). Repeated catching of the nesting male would also have been disruptive to the social dynamics at the nest, and thus inconsistent with our goal to measure the fitness consequences of natural and experimentally-induced variation in the neuroendocrine processes underlying male social interactions under natural conditions in the wild.

Quantitative Real-Time PCR

RNA was extracted from tissue punches using a Quick-RNA MicroPrep kit (Zymo Research) according to the manufacturer's instructions. To increase RNA yield, a Proteinase K digestion was performed by adding 20 µl of Proteinase K (20mg/ml) to tissue punches in RNA/DNA shield, and incubating at 55°C for 2 hours prior to performing the extraction. cDNA

was synthesized using the GoScript Reverse Transcription system (Promega) using random primers and oligo(dT). qPCR primers (Table 2) for the androgen receptor (AR), estrogen receptor α (ERα), estrogen receptor β (ERβ), elongation factor-1 (EF1) and GTP binding protein (GTPbp) were designed against sequences in the *S. ocellatus* transcriptome (Nugent *et al.* 2016). To generate primers for the vasotocin 1a receptor (V1aR), previously published degenerate primers designed to consensus sequences from the bluehead wrasse *v1a2* (Lema *et al.* 2012) were used to amplify *S. ocellatus* whole brain cDNA. The PCR product was purified and sequenced at the University of Texas at Austin DNA Sequencing Facility. Primers for arginine vasotocin (AVT) were designed against the complete coding sequence of the bluehead wrasse (*Thalassoma bifasciatum*; AY167033.1). Whole brain cDNA from *S. ocellatus* was used as a template for touchdown PCR. PCR products were submitted for sequencing at the University of Texas at Austin ICMB DNA Sequencing Facility. BLAST search of the resulting sequences confirmed their close homology with AVT and V1aR in over 100 other species. AVT was not detected in the *S. ocellatus* VMH.

Transcript expression was quantified in triplicate for each gene on a ViiA7 Real-time PCR System (Life Technologies) using GoTaq qPCR Master Mix (Promega). Standard curves for each gene were generated from serial dilutions of purified PCR products for each gene. Following the cycling protocol, continuous fluorescence was measured to generate a melting curve from 60°C to 95°C. ViiA7 software automatically generates baseline and threshold values for each gene, and the threshold cycle (Ct) values for each sample were used to determine cDNA quantity. Primer amplification efficiencies and relative expression levels were determined using MCMC.qpcr Bayesian analysis package in R (Matz *et al.* 2013), with GTP binding protein (GTPbp) and elongation factor 1 (EF1) as control genes. Data were analyzed by two-tailed t-test or Pearson's product-moment correlation in R (R Development Core Team 2011) using $\alpha < 0.05$ as the criteria for statistical significance between gene expression levels in flutamide and vehicle-injected groups (more details below).

Nest Collection and Paternity Analysis

Following nesting male capture, the algae from the nests of all twenty nesting males included in this study were collected and placed in individual plankton nets (Fieldmaster 153 um, 8-inch diameter Student Plankton Nets). These plankton nets were closed at one end with a cable tie; the other end had small corks (added to ensure that they remained upright in the water column) and a sample bottle that was changed daily. These nets were secured to a weighted line at ~3 meters depth and held in the wild for daily collection of hatched larvae (until larvae no longer emerged from the nest). Fertilized eggs of this species can develop normally as long as they are aerated and protected from predators. The above-described plankton net setup allows for both aeration and protection of the developing larvae, which hatch synchronously at night (Lejeune 1985). Nesting male reproductive success can be readily assessed by counting the total offspring hatched from his nest (Alonzo & Heckman 2010). For a subsample of these larvae, parentage was assigned using six polymorphic microsatellite loci as previously described (Alonzo & Heckman 2010). The number of alleles per locus is 17-37 (mean=25.0), and expected heterozygosity ranged from 0.73 to 0.90 (mean=0.87). Combined non-exclusion probability for the first parent is 0.00185. Nesting males were assigned as the father based on strict exclusion (if a male did not share an allele at each locus that could be compared, he was excluded as the father). Only larvae from days 4-6 days following nest collection were included in the paternity analyses as they represent the offspring arising from post-injection spawning based on minimum development times. For each nest, either 45 (15 per collection day) or all (if fewer than 45) larvae were genotyped for paternity.

Sperm Characterization

A separate cohort of 17 nesting males were acclimated to large (~250 liter) holding tanks in the field station laboratory supplied with running seawater, rocks and algae collected at the field site. These males were injected with either vehicle (n=7) or flutamide (n=10) as described above. Sperm were collected three days after injection, to mimic the timing of the

behavioral experiment conducted in the wild. By pressing gently along the male's abdomen toward the genital pore, we collected 2 μl of milt from each male and activated this sperm sample in 1 mL seawater in one chamber of a 6-well plate. A 2 μl subsample of the activated sperm was then added to the chamber of a 20 μm, four-chamber Leja slide and videotaped until no sperm cells were motile under 10x negative phase contrast using Motic BA310 light microscope and 60 Hz EIA monochrome RS-170 camera for later analysis of sperm characteristics. Using these videos, we determined sperm concentration, velocity and motility for each male 40 seconds after activation of the sperm, using a Hamilton Thorne CEROS CASA system. Forty seconds post-activation was the earliest time point at which all of the samples could be viewed under the microscope following activation such that the sperm characteristics could be measured. Fish were returned to the wild immediately following milt collection.

Statistical Analyses

Our analyses of the behavioral data focus first on examining whether there was a significant effect of treatment on the change between the pre- and post-manipulation observations. Data comparing changes in behavior across flutamide and vehicle-injected nesting males were therefore analyzed by calculating the difference scores between the pre-observations and the averaged post-observations, and conducting independent groups t-tests (on variables with normal distributions) or Mann-Whitney U-tests (on variables with non-normally distributed data) or Pearson's product-moment correlation in R (R Development Core Team 2011) using $\alpha < 0.05$ as the criterion for statistical significance. Other variables such as hormone levels and sperm characteristics were analyzed by two-tailed t-test in R (R Development Core Team 2011) using $\alpha < 0.05$ as the criteria for statistical significance (more details below). We also examined whether there was a statistical association between the experimentally-induced variation in neuroendocrine mechanisms, social interactions and mating patterns, using a Pearson's product-moment

correlation in R (R Development Core Team 2011) and $\alpha < 0.05$ as the criterion for statistical significance. The aim of these analyses was not to quantify the selection gradient (sensu Lande & Arnold 1983) on specific neuroendocrine patterns. Instead, the goal was to determine the potential for sexual selection on the mechanisms underlying male social interactions during reproduction. We therefore focused on how experimentally induced variation in neuroendocrine mechanisms affected mating patterns and reproductive success (Linnen & Hoekstra 2009). In particular, we focused on measuring whether the change in mating success is associated with variation in a trait of interest. This can be used to characterize the potential for sexual selection on that trait (Andersson 1994).

RESULTS

Our experimental manipulation of AR signaling affected male reproductive physiology, gene expression in the brain, social interactions among males, and male mating success as described in each corresponding section below.

1) Androgen receptor antagonism reduces 11-ketotestosterone and alters gonadal function

We first considered how experimentally manipulating androgen receptor signaling affected circulating hormones and male reproductive physiology. Nesting males are socially dominant among male *S. ocellatus* morphs, with significantly higher basal 11-ketotestosterone levels relative to sneaker and satellite males (From Stiver *et al.* 2014: Nesting Male: NM: mean = 0.500 ng/mL, SEM = 0.088, N = 12; Satellite male: mean = 0.152 ng/mL, SEM = 0.034, N = 12; Sneaker male: mean = 0.088 ng/mL, SEM = 0.013, N = 12). Compared to vehicle-treated controls, wild nesting males that received a single injection of flutamide had significantly lower levels of circulating 11-ketotestosterone three days post injection (Fig. 1A; $t(10.68) = 3.055$, $p = 0.011$; vehicle-treated males: mean= 52.1 pg/mL, SEM= 10.7; flutamide-treated males: mean= 17.75 pg/mL, SEM= 3.14). The experimentally-reduced 11-ketotestosterone levels (shown in Figure 1A) were, however, in the natural range

for males in this species, falling slightly below natural levels of circulating 11-ketotestosterone of sneaker and satellite males (Stiver *et al.* 2014). The experimental manipulation therefore successfully altered androgen signaling, as predicted.

Moreover, fish treated with flutamide had a reduced gonadosomatic index (GSI, i.e. gonad mass/total body mass) compared to controls (Fig. 1B; $t(10.67) = 3.729$, $p = 0.004$). Gonadosomatic index was positively correlated with plasma 11-ketotestosterone (Fig. 1C; Pearson's $r(15) = 0.623$, $p = 0.008$) as previously reported in teleost fish (Zeyl *et al.* 2014). 11-ketotestosterone is a well-known stimulant of spermatogenesis in fish (Miura *et al.* 1991; Borg 1994; Cavaco *et al.* 1998, 2001; Schulz *et al.* 2010; Zeyl *et al.* 2014), thus in addition to reduced 11-ketotestosterone production, we predicted that sperm production and characteristics might also be impaired by flutamide administration. A laboratory study investigating the impact of AR blocking and reduced 11-ketotestosterone on sperm characteristics revealed a significant decrease in sperm velocity (Fig. 1D; $t(13.33) = 2.296$, $p = 0.038$) and a non-significant trend toward decreased sperm count in flutamide treated nesting males compared to controls in nesting males maintained in otherwise identical conditions in the lab (Fig. 1E; $t(6.53) = 2.074$, $p = 0.079$). On these same fish, there was no significant effect of flutamide on nesting male sperm motility (Fig. 1F; $t(10.56) = -1.140$, $p = 0.279$).

2) Androgen receptor antagonism alters brain hormone receptor mRNA expression

We next document how experimental manipulation of AR signaling affected neural gene expression. Because of their well-known role in social and sexual behaviors and their sensitivity to feedback by circulating gonadal hormones, we quantified levels of AR, estrogen receptor α (ERα), estrogen receptor β (ERβ), and vasotocin 1a receptor (V1aR) in the preoptic area (POA; Fig. 2A,B) and ventromedial hypothalamus (VMH; Fig. 2A,I). These brain regions are critical for male reproductive behavior and territorial aggression (Heimer & Larsson 1967; Olivier 1977; Arendash & Gorski 1983; Koyama *et al.* 1984; Nelson &

Chiavegatto 2001). In the POA, AR (Fig. 2C; $t(14.78) = 6.905$, $p < 0.001$) and V1aR (Fig. 2F; $t(17.19) = 6.355$, $p < 0.001$) expression levels were significantly reduced by flutamide injection. We also found a correlation between V1aR mRNA levels in the POA and circulating 11-ketotestosterone (Fig. 2G; Pearson's $r(17) = 0.602$, $p = 0.006$), consistent with a role for androgens in controlling the expression of this critical receptor for social and reproductive behaviors (Young *et al.* 2000). AR antagonist treatment did not significantly alter AR or V1aR in the VMH or ERα or ERβ mRNA levels in either the POA or VMH (Fig. 2D-E, J-M). We found high levels of variation in AVT mRNA expression in the preoptic area in both control and flutamide-treated nesting males with no significant differences in AVT levels between experimental groups (Fig. 2H). AVT is present at very low levels in the teleost VMH (Greenwood et al. 2008; Rodriguez-Santiago *et al.* 2017), and our quantification was too low to reliably detect the gene in this region.

3) Androgen receptor antagonism in nesting males alters social dynamics at the nest

As described above, AR antagonist treatment affected circulating 11-ketotestosterone levels, nesting male reproductive physiology, and AR expression in the POA, a region critical for male reproductive behaviors. We would therefore also expect nesting males treated with flutamide to exhibit marked changes in social and sexual behavior compared to their pre-injection baseline behavior and compared to vehicle-injected control males. Specifically, we predicted decreases in androgen-driven behaviors, such as courtship, aggression and nest defense in flutamide-treated males.

Surprisingly, however, there were no significant effects of treatment on courtship, parental care, or total nesting male aggression (Fig. 3A-C; Table 1). Instead, further analysis of our behavioral data revealed subtle but important effects of AR inhibition on social interactions with conspecific males. Nesting males in both treatment groups showed decreased aggression post-injection (Table 1), which likely results from a decrease in the number of sneaker males at nests post-injection (Table 1). However, following treatment,

aggression by flutamide-treated nesting males, but not control nesting males, was biased towards *satellite males* (Fig. 3D; Table 1). Furthermore, while sneaker males generally showed decreased submission to the nesting male in the post-observations, this decrease in submission was significantly greater at those nests where the nesting male had received a flutamide injection (Fig. 3E; Table 1). These findings are reflected in the fact that changes in sneaker-directed aggression by nesting males (pre and post flutamide injection) was positively correlated with changes in the number of submissions received by nesting males (Fig. 3F; $r(21) = 0.551$, $p = 0.006$), implying that nesting male aggression determines the degree of conspecific submission and that variation in androgen signaling in dominant animals can alter the social dynamics within the group. These patterns demonstrate a significant change in the social behavior of male conspecifics arising from the manipulation of nesting males.

In addition, we found that variation in V1aR expression, and variation in expression of its ligand AVT, in the POA were linked to variation in nesting male behavior. Nesting males with lower POA V1aR mRNA expression (generally flutamide-treated) displayed more aggression toward satellite males as opposed to sneakers following AR antagonist treatment (Fig. 3H; $r(16) = -0.688$, $p = 0.0016$). Although there were no significant effects of flutamide treatment on AVT mRNA expression in the POA (Fig. 2F), among-male variation in AVT expression in the POA was correlated positively with a change in courtship (Fig. 3I; $r(13) = 0.656$, $p = 0.007$).

4) Androgen receptor antagonism and male mating success.

Having found significant effects of flutamide injection on male physiology and behavior (as described above), we next consider whether these changes were associated with changes in male mating or fertilization success. In this species, males naturally exhibit striking variation in both social behavior and mating success both within and between alternative male types. Due to this natural variation among males, the best measure of the

fitness consequences of the experimentally-induced variation in the mechanisms underlying male social behavior is a change in a fitness measure following the manipulation. A direct pre/post manipulation comparison is only possible for mating success because measuring fertilization success and paternity requires collecting the entire nest. We therefore focus our analyses and discussion primarily on changes in mating (i.e. spawning) success. However, due to the observed changes in nesting male sperm production and characteristics and the potential for changes in parental care, we also examined nesting male paternity, total fertilization success and number of larvae emerging from the nest.

As above, we focus on two kinds of analyses. First we asked if the treatment (vehicle versus flutamide injection) had a significant effect on the variable of interest (e.g. change in mating success, paternity or number of larvae emerging from the nest). We did not find a significant effect of flutamide injection on the change in nesting male spawning rate (Table 1, Fig. 4A). Despite the hormonal, neural gene expression, and behavioral changes observed in nesting males following flutamide treatment, we also found no significant effects of the manipulation on the number of emergent larvae per day (Fig. 4E; $U_{(10,13)} = 46.5$, $p = 0.257$) and total larvae (Fig. 4F; $U_{(10,13)} = 52.5$, $p = 0.446$) or in the estimated number of larvae sired by nesting males as determined by microsatellite paternity analysis (Fig. 4G; $U_{(5,5)} = 8.00$, $p = 0.421$).

Second, we asked if natural and experimentally-generated phenotypic variation in neuroendocrine mechanisms was associated with variation in mating success, paternity or number of larvae emerging from the nest. We also asked whether the experimentally-induced variation in gene expression and circulating hormones are associated with variation in the change in mating success. We found that AR mRNA expression in the POA was positively correlated with the change in mating success following experimental manipulation (Fig. 4C; $r(18) = 0.47$, $p = 0.036$). We also found that among-male variation in AVT in the POA was correlated negatively with nesting male mating success (Fig. 4D; $r(16) = -0.510$, p

= 0.031) when post-injection mating rate is normalized for pre-injection among-nest differences in mating success. Though AVT mRNA in the POA was not significantly altered by AR inhibition (Fig. 2F), we found a positive correlation between AVT in the POA and the number of emergent larvae per day (Fig. 4H; $r(16) = 0.612$, $p = 0.006$) and the proportion of larvae sired by the nesting males (estimated based on differences in paternity, Fig. 4I; $r(5) = 0.952$, $p = 0.0009$). Finally, changes in sneaker submissive behaviors directed toward the nesting male (during the pre- versus post-injection observation; see Table 1) were associated with significant changes in the nesting male mating success (Fig. 4B; $r(20) = 0.494$, $p = 0.019$), such that nesting males with a greater decrease in mating success (generally flutamide-treated males) also received relatively fewer submissions post treatment.

DISCUSSION

There is an intuitive relationship between the mechanisms underlying variation in reproductive traits and their evolutionary consequences. Reproductive behaviors therefore provide a powerful opportunity to study how social interactions arise from the interplay between mechanistic, behavioral and evolutionary processes. Here we report the physiological, behavioral, and reproductive effects of variation in neurogenomic and neuroendocrine signaling in a wild fish with male alternative reproductive tactics. Although several studies have assessed the impact of hormone levels on reproductive fitness (e.g. Hegner & Wingfield 1987; Ketterson et al. 1992; Moss *et al.* 1994; Hunt *et al.* 1999; Moreno *et al.* 1999; Van Duyse *et al.* 2000; De Ridder *et al.* 2000; Alonso‐Alvarez 2001; Peters *et al.* 2002; Clotfelter *et al.* 2004; Van Roo 2004; Hunt & Wingfield 2004; Mougeot *et al.* 2005; Mills et al. 2007, 2008, 2009; McGlothlin *et al*. 2007, 2008, 2010; Veiga & Polo 2008), we are not aware of any other experiments conducted under natural conditions in the wild linking changes in hormone signaling to variation in neural gene expression to behavior and fitness consequences in free-living vertebrates. We manipulated AR activity to better understand the role of natural variation in gonadal steroid hormone signaling in shaping the social and

reproductive behaviors in wild *S. ocellatus*, a species with marked within-sex differences in circulating 11-ketotestosterone and male behaviors. We found that manipulating a single individual at each nest had cascading behavioral effects not only on the focal individual but also for the other males at the nest.

Previously, systemic treatment with the androgen antagonist flutamide was shown to decrease overall aggression in and enhance courtship behaviors in bluegill sunfish, another species that displays paternal care (Rodgers *et al.* 2013). In contrast, *S. ocellatus* nesting males showed no changes in total aggressive or courtship behaviors, however nesting males treated with flutamide directed less aggression toward sneakers and more toward satellite males, their cooperative allies in nest defense and courtship (Stiver & Alonzo 2013). In addition, flutamide-treated nesting males received fewer submissive behaviors from sneaker males perhaps as the result of their decreased sneaker-directed aggression. Thus, our results suggest that reduced androgen signaling in dominant nesting males might have effects on nesting male dominance and social interactions, affecting behavioral traits critical for nesting male fitness and that AR inhibition in a single member of the social group can alter *S. ocellatus* social dynamics. We did not find a direct effect of the manipulation on male mating success, paternity or larval production, but we did find that decreased sneaker male submissiveness was significantly correlated with decreased nesting male mating success.

Variation in nesting male androgen signaling may also affect the reproductive success of females choosing that nest site (and nesting male), given that females are known to prefer mating with nesting males (Alonzo & Warner 2000; Alonzo 2004; Alonzo & Heckman 2010). Natural variation in androgen signaling also has the potential to drive variation in reproductive success through variation in reproductive physiology (i.e. sperm characteristics) and variation in social interactions, with potential fitness consequences for all individuals breeding at the nest.

Following behavioral observations, we quantified the expression of known mediators of social and sexual behaviors in key regions of the social decision making network (SDM) (O'Connell & Hofmann 2012), the complex of brain nuclei regulating key behaviors for survival and reproductive fitness. We identified a correlation between 11-ketotestosterone levels and V1aR mRNA expression in the POA, both of which were markedly decreased in flutamide-treated nesting males. This finding supports the well-documented role of androgens in V1aR regulation and suggests that androgen-mediated regulation of the vasotocin receptor system is brain-region specific in teleosts, as we did not observe changes in V1aR in the VMH. Importantly, we found that mRNA levels of V1aR in the POA negatively correlated with increased satellite-directed aggression in nesting males. Androgen-induced decreases in V1aR expression may provide a plausible biological mechanism for the change in the direction of nesting male aggression (from sneaker to satellite males). V1aR is a critical mediator of social recognition in several mammalian species (Landgraf *et al.* 2003; Bielsky & Young 2004; Winslow & Insel 2004; Bielsky *et al.* 2005; Albers 2012) and V1aR knockout in mice demonstrates that this receptor is essential for recognition of individual conspecifics (Bielsky *et al.* 2004). Thus, reduction of androgen signaling in wild nesting males leading to decreases in POA V1aR expression may have produced impairments in social recognition in these fish. Additional mechanistic studies are needed to determine if changes in POA V1aR are responsible for social status, recognition and/or cognition in *S. ocellatus.*

Although we did not directly manipulate the vasotocin system in these studies, our data support an important role for V1aR signaling in reproductive outcomes in the wild. We found no significant changes in POA expression of AVT, V1aR's ligand, between control and flutamide-treated nesting males, which is consistent with previous findings that AVT levels are largely dependent on social status and not necessarily on circulating androgens (Semsar & Godwin 2003). However, we found that variation in AVT mRNA levels in the POA correlated positively with courtship and negatively with changes in mating (spawning)

success. The correlation of AVT with courtship behaviors is consistent with previous studies demonstrating that AVT increases courtship among a diverse group of species (Santangelo & Bass 2006; Huffman *et al.* 2015) including another Labridae species, the bluehead wrasse (Semsar *et al.* 2001). Our observation that variation in AVT expression correlated negatively with spawning behavior is contrary to reports in other species in which exogenous AVT enhances spawning (Pickford & Strecker 1977) or in which dominant, reproductive males have greater levels of AVT in the POA than subordinate or non-reproductive individuals (Godwin *et al.* 2000; Aubin-Horth *et al.* 2007; Kleszczyńska *et al.* 2012). However, in the ocellated wrasse we previously found that spawning and courtship negatively covary (Alonzo 2008). Successful nesting males engage in less courtship than unsuccessful males. This may be because of female mate choice copying where successful males don't "have" to court to get females to spawn at their nests (Alonzo 2008). This intriguing aspect of *S. ocellatus* behavior may explain why AVT is positively associated with courtship but negatively associated with spawning. Perhaps AVT does not drive spawning behaviors in this species. Further mechanistic investigations of AVT signaling are necessary to determine its exact function.

11-ketotestosterone manipulation studies in other teleosts suggest that the role of this androgen may vary greatly across species, highlighting species specificity in hormonal control of behavior. For example, some studies have reported that 11-ketotestosterone increases aggressive behaviors in fish (Borg 1994; Oliveira & Canário 2000), whereas others have reported decreases in aggression following 11-ketotestosterone administration (Oliveira *et al.* 2001b). Implants of 11-ketotestosterone fail to induce courtship in non-territorial, parasitic breeding plainfin midshipman (Remage-Healey & Bass 2007) despite consistent findings that elevated 11-ketotestosterone is associated with courtship in several other teleost species (Kindler *et al.* 1989; Sikkel 1993; Pankhurst *et al.* 1999; Salek *et al.* 2001; Páll *et al.* 2002). Somewhat surprisingly, in the current study large decreases in 11-ketotestosterone did not directly correlate with changes in behaviors previously associated

with gonadal hormone signaling (i.e. spawning, courtship, total aggression). Instead, androgen manipulation resulted in a change in the direction of nesting male aggression away from parasitically-spawning sneaker males to their cooperative allies, satellite males.

AR and V1aR mRNA levels in the POA were reduced in nesting males administered flutamide relative to controls. The activity of both of these receptors in this region is critical for male reproductive behaviors, aggression and territorial behaviors (Insel & Young 2000; Goodson & Bass 2001; Burmeister *et al.* 2007; Donaldson & Young 2008; Insel 2010). However, we found that reduced levels of AR mRNA in the POA correlated with decreased spawning. We found no changes in AR or V1aR in the VMH and no changes in ERα or ERβ in either region. These findings are consistent with numerous studies in mammals showing that region-specific AR signaling plays an important role in determining reproductive behaviors and underscores the importance of the POA in expression of male sexual behaviors across vertebrates (Heimer & Larsson 1967; Arendash & Gorski 1983; Koyama *et al.* 1984).

As described above, testicular androgens are key regulators of male social and sexual behaviors through their central activation of brain regions within the SDM and their mediation of neural gene expression patterns. In the current study, AR antagonist treatment dramatically reduced plasma levels of 11-ketotestosterone in wild *S. ocellatus* nesting males. In teleost fish, 11-ketotestosterone is produced by the testes (Idler & MacNab 1967), induces spermatogenesis (Miura *et al.* 1991), and is critical for gonadal morphology and function (Reinboth 1975). We administered flutamide systemically, via intraperitoneal (IP) injection, and therefore the decreases we observed in circulating 11-ketotestosterone were likely the result of androgen receptor inhibition in the testes. This suggestion is congruent with our findings that flutamide-treated nesting males had large decreases in gonadosomatic index (i.e. gonad mass/total body mass) and sperm velocity as compared to controls following flutamide treatment. Flutamide also likely acted centrally to produce these marked changes

in reproductive physiology through alterations in neuroendocrine signaling. In other teleost species, exogenous androgens have been shown to increase the number and activity of pituitary gonadotropes (Schreibman *et al.* 1986) and gonadotropin releasing hormone (GnRH) administration enhances sperm production, milt volume, and sperm motility (Clearwater & Crim 1998). Although we did not measure GnRH in the current study, we predict that reduced levels of circulating 11-ketotestosterone in flutamide-treated nesting males likely decreased GnRH production and/or release leading to impairments in gonadal function in nesting males. Overall, our data confirm previous findings that AR signaling is necessary for normal gonadal functioning in male teleost fish and demonstrate that a single injection of flutamide can have large effects on the reproductive behavior and physiology of *S. ocellatus* nesting males. These findings also suggest that natural variation in 11-ketotestosterone among male morphs could impact reproductive physiology and consequently reproductive strategy and success. Increased sperm counts and velocity are known to enhance paternity in this species (Alonzo *et al.* 2016), meaning variation in AR signaling could lead to variation in male fertilization success (when in sperm competition).

Despite our findings that AR inhibition altered fitness-related traits such as sperm characteristics and social interactions, we did not directly observe statistically significant changes in paternity. This may have been due to the timing and duration of our treatment and/or the variability inherent in studies of wild populations (as paternity can only be measured by collecting the entire nest in this species we are not able to look at changes in paternity by comparing paternity before and after the injection). This variance in fertilization success is not simply noise; instead it represents the potential for selection under natural conditions in the wild, particularly in species in which reproductive competition is intense making the potential for sexual selection among males high. For instance, in the 20 nesting male nests collected for this study (10/treatment), the number of larvae ranged from 0 to over 4000. We controlled for nest activity by including only nests with active spawning, a satellite, and at least two sneakers during initial baseline observations. However, our

behavioral quantifications at each nest were conducted over the course of three days, with reproductive activity at each nest variable and changing among days. In addition, predation of eggs and larvae in nests could further contribute to the variability in our data. Despite this, we did find that variation in AVT expression correlated with the number of offspring in the nest as well as the proportion of nesting-male sired offspring. This is intriguing in light of our finding that AVT was also associated with decreases in spawning behavior in nesting males.

Manipulating androgen signaling in freely behaving *S. ocellatus* nesting males demonstrated that natural variation in androgen signaling regulates reproductive physiology, neural gene expression and social interactions critical for reproductive success in the wild. This provides direct evidence that variation in androgen signaling alters behavioral interactions under natural conditions. This experimentally-induced variation in focal male social and reproductive behavior was also found to have cascading consequences for the social behavior of the others in the same social group. Recent research has shown that the fundamental neural and hormonal "building blocks" underlying social and reproductive behavior are highly conserved across vertebrates (O'Connell & Hofmann 2012; Kalueff *et al.* 2014), making a wider range of species relevant models for understanding the origins and mechanisms underlying vertebrate behavior, including humans. These results identify key mechanisms likely underlying variation (and potentially plasticity) in social and reproductive behaviors. Our results reveal the potential for sexual selection to be operating on the neuroendocrine mechanisms underlying these behaviors and social interactions. This study therefore represent a critical first step in understanding how the evolution of these mechanisms may influence and explain the striking diversity of social and reproductive systems observed among vertebrates living under natural conditions in the wild.

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DATA AVAILABILITY

Raw data for 11-kt, sperm characteristics, GSI, gene expression, behavior and larval counts are available in Dryad (doi:10.5061/dryad.v8c7f47).

AUTHOR CONTRIBUTIONS

BMN and SHA jointly conceived of and planned the study. BMN, KAS and SHA designed and performed the field experiments and made the behavioral recordings in the field. BMN and HAH designed and developed the methods for the hormone and gene expression assays and analyses. BMN performed the hormone and gene expression assays and analyses. SHA performed the sperm analyses. KAS quantified and analyzed behavioral and paternity data. BMN and KS performed all statistical analyses. BMN wrote the first draft of the manuscript, and all authors contributed to the writing of the final manuscript.

FIGURE LEGENDS

Fig. 1: Androgen receptor antagonism alters reproductive physiology in wild *S. ocellatus* nesting males. A,B. Three days after flutamide injection, circulating 11-ketotestosterone (n=9 flutamide, n=10 vehicle) and gonadosomatic index (n=10 flutamide, n=8 vehicle) were significantly decreased compared to vehicle-treated controls. Though we conducted 10 replicates per treatment, slightly different sample sizes arose when problems occurred during sample collection in the field (e.g. insufficient blood or inability to collect the entire gonad). **C.** gonadosomatic index (GSI, i.e. gonad mass/total body mass) and 11-ketotestosterone levels showed a significant correlation (open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression). **D.** Sperm velocity was significantly decreased three days after flutamide treatment (n=10 flutamide, n=7 vehicle). **E,F.** Flutamide treatment did not cause a statistically significant decrease in sperm count in nesting males compared to vehicle controls (n=10 flutamide, n=7 vehicle) and did not alter the percentage of motile sperm sampled (n=10 flutamide, n=7 vehicle). Box plots represent data median (center line), upper and lower quartiles (box limits), and 1.5x interquartile range (whiskers). *p < 0.05, **p<0.01. Note: Panels A, B and C present data collected on males released back into the wild, while panels D, E and F represent data collected on nesting males kept in the lab.

Fig. 2: Flutamide produces region-specific changes in hormone receptor expression. **A.** Illustration depicting the location of coronal section collection for isolation of the POA and VMH. **B.** Illustration of location of POA tissue punch collection. In the preoptic area (POA, top), flutamide significantly reduced androgen receptor (**C**, n=10 flutamide, n=10 vehicle) and vasotocin 1a receptor (**F**, n=10 flutamide, n=10 vehicle) mRNA expression. **G**. Vasotocin 1a receptor expression in the POA correlated with circulating 11-ketotestosterone (11-KT) levels (open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression). Flutamide had no effect on mRNA levels of estrogen receptor α (**D**, n=9 flutamide, n=10 vehicle), β (**E**, n=10 flutamide, n=10 vehicle), or arginine vasotocin (**H**, n=8 flutamide, n=10 vehicle) in the POA. **I.** Illustration of location of VMH tissue punch collection. In VMH, flutamide treatment had no effect on androgen receptor (**J**, n=9 flutamide, n=9 vehicle), estrogen receptor α (**K**, n=9 flutamide, n=10 vehicle), estrogen receptor β (**L**, n=9 flutamide, n=10 vehicle), or vasotocin 1a receptor (**M**, n=9 flutamide, n=10 vehicle) mRNA levels. Box plots represent data median (center line), upper and lower quartiles (box limits), and 1.5x interquartile range (whiskers). Open circles on boxplots represent statistical outliers. ***p<0.0001

Fig. 3: Nesting male androgen receptor inhibition changes social dynamics at the nest. Flutamide did not significantly alter nesting male courtship (**A**, n=10 flutamide, n=10 vehicle), parental behaviors (**B**, n=10 flutamide, n=10 vehicle), or total aggression (**C**, n=10 flutamide, n=10 vehicle) compared to vehicle-treated animals. **D**. The proportion of nesting male aggression was shifted from sneakers to satellites in flutamide-treated nesting males. Flutamide-treated nesting males also received fewer submissive behaviors from sneakers at their nests (**E**, n=10 flutamide, n=10 vehicle). Data are represented as baseline observation values compared to the average of 3 post-injection observations; for the actual test, the pre-post difference scores of the two treatment groups were compared. **F.** The number of submissions received by lower-ranking males correlated with sneaker-directed aggression (open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression). **G.** The risk of sperm competition (i.e. proportion of female spawns that involve not only the nesting male but also sperm competition from sneakers or satellite males) did not change significantly as a result of flutamide injection**. H.** POA V1aR mRNA expression correlated with baseline-corrected proportions of satellite-directed

aggression. **I.** POA AVT mRNA levels were associated with increased courtship in nesting males post injection (open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression). Box plots represent data median (center line), upper and lower quartiles (box limits), and 1.5x interquartile range (whiskers). Open circles on boxplots represent statistical outliers. Change in behavior represented in scatterplots was calculated by averaging the behavior scores on the 3 days post-injection and subtracting the pre-injection behavior score (B, C, D).

Fig. 4: Nesting male androgen receptor signaling and fitness-related outcomes. **A**. Flutamide did not significantly alter nesting male mating (i.e. spawning) rate (n=10 flutamide, n=10 vehicle). **B** The change in mating rate was however significantly correlated with submissions received by lower-ranking males (open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression). **C.** POA androgen receptor expression correlated with the change in mating rate (open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression), and the change in nesting male mating rate also was negatively correlated with POA AVT expression (**D.**). There were no differences in the number of larvae per day (**E**, n=10 flutamide, n=10 vehicle) or total larvae in nest (**F**, n=10 flutamide, n=10 vehicle) counted in the nests of vehicle and flutamide-treated nesting males. **G**. There was also no significant difference in the estimated number of nesting male-sired larvae between groups (n=5 flutamide, n=5 vehicle). Although flutamide did not alter mRNA levels of AVT in the nesting male POA, variation in AVT expression was highly correlated with the proportion of larvae sired by nesting males (**H**, open circles = vehicle, filled circles = flutamide, dotted lines represent 95% confidence interval from linear regression) as well as the average number of larvae in the nest per day (**I**). Box plots represent data median (center line), upper and lower quartiles (box limits), and 1.5x interquartile range (whiskers). Open circles on boxplots represent statistical outliers. Change in behavior represented in scatterplots was calculated by averaging the behavior scores on the 3 days post-injection and subtracting the pre-injection behavior score (F, H, I).

Table 1a: A summary of behavioral variables. Tests are either unpaired t-tests, or Mann-Whitney U tests for those variables where data was not normally distributed.

Variable tested	***Test statistic***	***p-value***	**Interpretation**
Courtship	t = -0.211	0.835	No difference
Parental care	t = 1.403	0.175	No difference
Average number of Accessory Males (satellites + sneakers)	t = -0.001	0.999	No difference
Nesting male aggression to satellite and sneakers	t = -0.157	0.877	No difference
Proportion of nesting male aggression directed at satellites versus sneakers** ($N_{control}$ = 12, $N_{flutamide}$ = 8)	**t = 2.265**	**0.036**	**Increased in aggression to satellite by flutamide treated males**
Sneaker submission to nesting male	**t = 12.080**	**0.048**	**Decrease in submission by sneakers to flutamide treated males**
Nesting male mating rate	t = -1.465	0.158	No difference
Number of sneak-spawnings by satellites and sneakers	U = 57.0	0.648	No difference
Proportion of mating events which included sneaking** ($N_{control}$ = 8, $N_{flutamide}$ = 7)	U = 20.5	0.397	No difference

**(Note – there is a slight sample size decrease in these analyses as males who did not perform the target behavior in both the pre- and post-observation could not be included in the proportional calculation)

Table 1b: The mean, standard deviation, and range of all behavior variables.

Variable	**Control treatment**		**Flutamide treatment**	
	Pre	**Post**	**Pre**	**Post**
Courtship	6.5 ± 5.2 0 – 18	3.0 ± 2.4 0 - 10	7.5 ± 4.8 3 - 17	3.5 ± 3.1 0 - 11
Parental care	7.9 ± 4.3 0 - 14	8.2 ± 4.5 0 - 19	8.0 ± 6.8 0 - 17	11.7 ± 4.0 0 - 35
Nesting male aggression to satellite and sneakers	20.2 ± 11.9 6 - 50	7.8 ± 7.9 0 - 34	22.1 ± 17.5 2 - 52	8.9 ± 9.4 0 - 45
Sneaker submission to nesting male	3.7 ± 3.8 0 - 13	1.6 ± 2.3 0 - 12	8.1 ± 6.8 0 - 23	2.1 ± 3.3 0 - 26
Nesting male mating rate	3.5 ± 3.1 0 - 12	2.3 ± 2.7 0 - 12	6.2 ± 5.3 0 - 16	2.1 ± 2.1 0 - 13
Number of sneak-spawnings by satellites and sneakers	0.9 ± 1.6 0 - 6	0.3 ± 0.4 0 - 4	1.7 ± 2.8 0 - 9	0.3 ± 0.5 0 - 5

Table 2: PCR primer sequences and efficiencies

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Efficiency
AR	Forward	TGCGAGATAACTGCTGGTCA	173	1.90
	Reverse	ATGACTCCTGCTCGTTTCCT		
ERα	Forward	TGGGATGCTAAAAGAGGGA	200	1.99
	Reverse	GTCGGGCATGGCAAATAACT		
ERβ	Forward	GAGGCACAGTCCGAAATTCC	208	1.94
	Reverse	TCCTCCAGTCCAGAAAGTG		
V1aR	Forward	GGAATGAGGAGGTGGCTCAA	150	2.02
	Reverse	CCAGGCTCAGGTGTTTGATG		
AVT	Forward	TACATCCAGAACTGTCCCCG	191	2.04
	Reverse	GGGGTGAGCAGGTAGTTCTC		
EF1	Forward	ATGAATCACAAACAGGGCCG	184	1.93
	Reverse	CTGCAGGTGGATGAAGAACG		
GTPbp	Forward	GGGCATTTTGTTCCACCGAT	151	1.94
	Reverse	ATGAAGCGGAAGTGGACTGA		

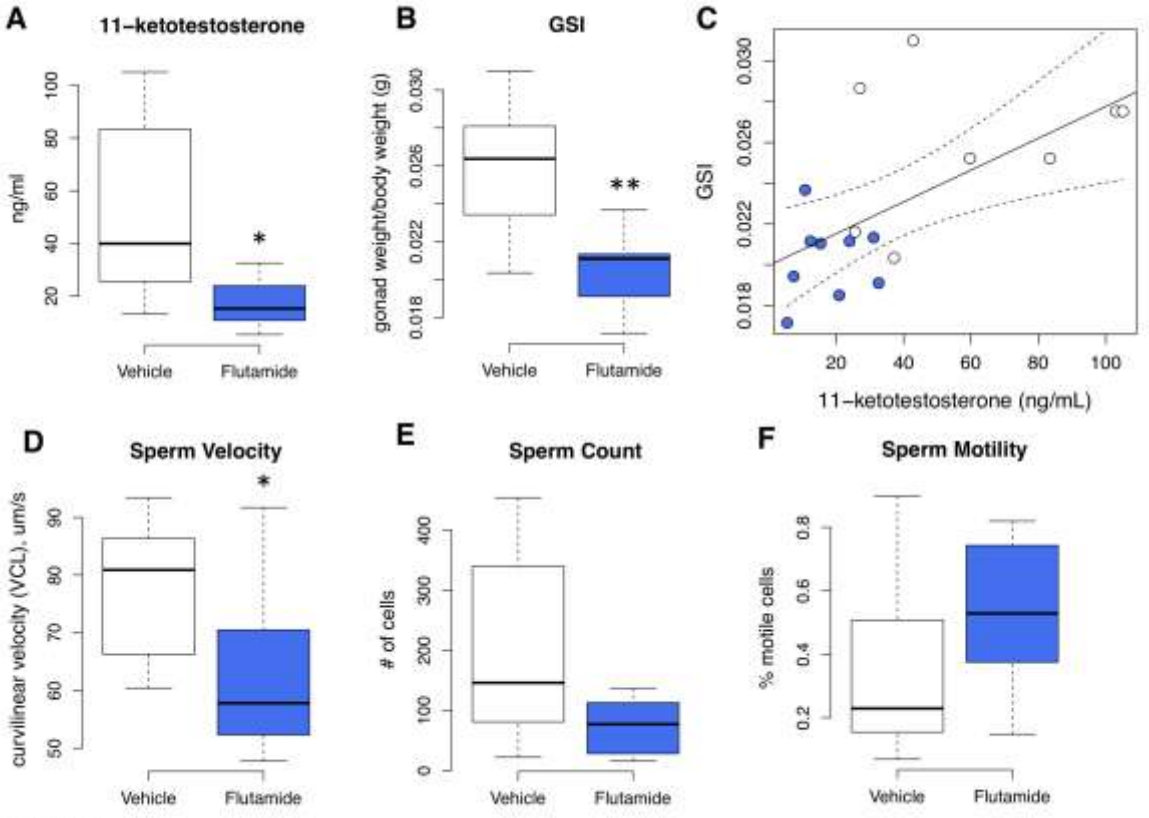

Figure 1

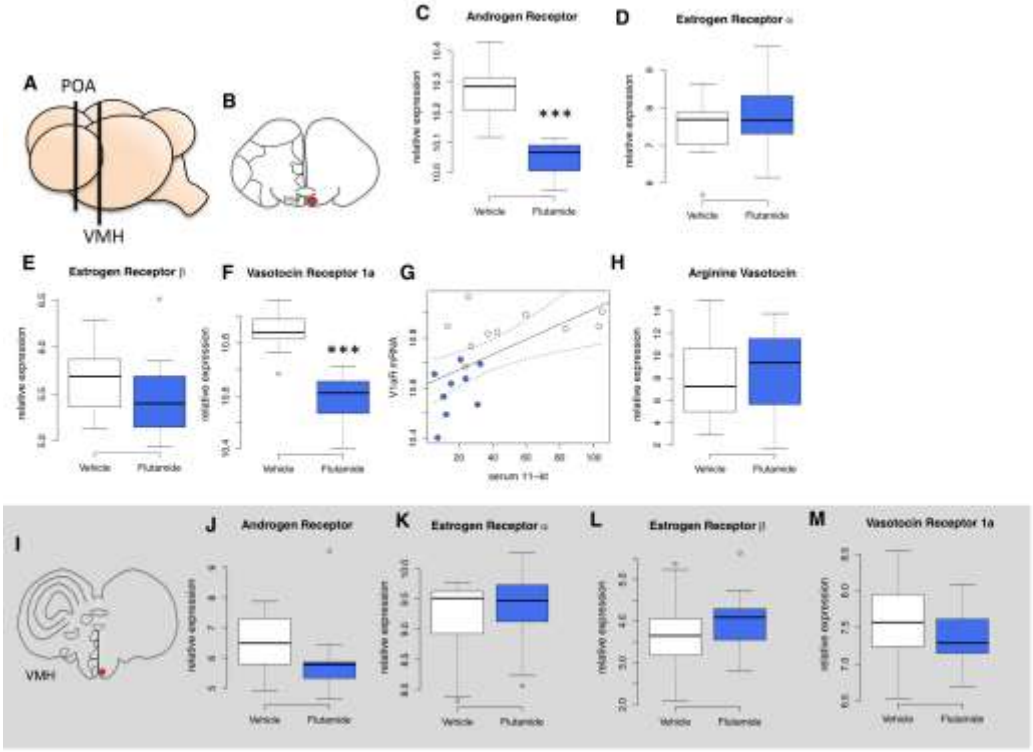

Figure 2

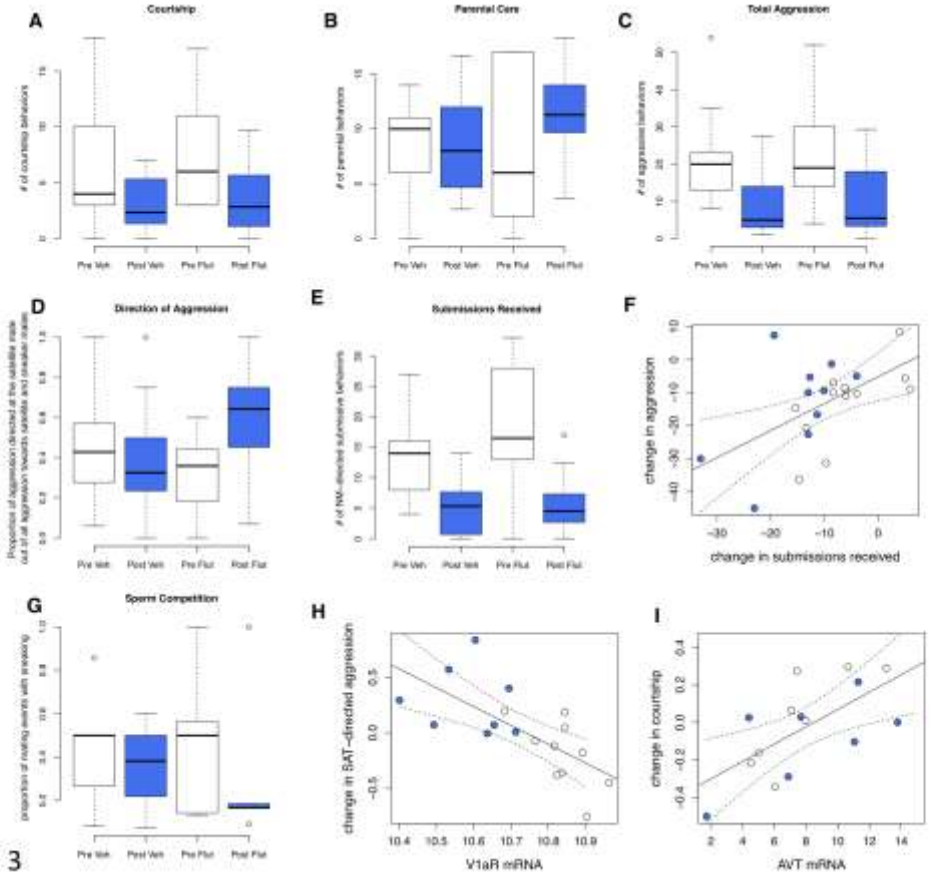

Figure 3

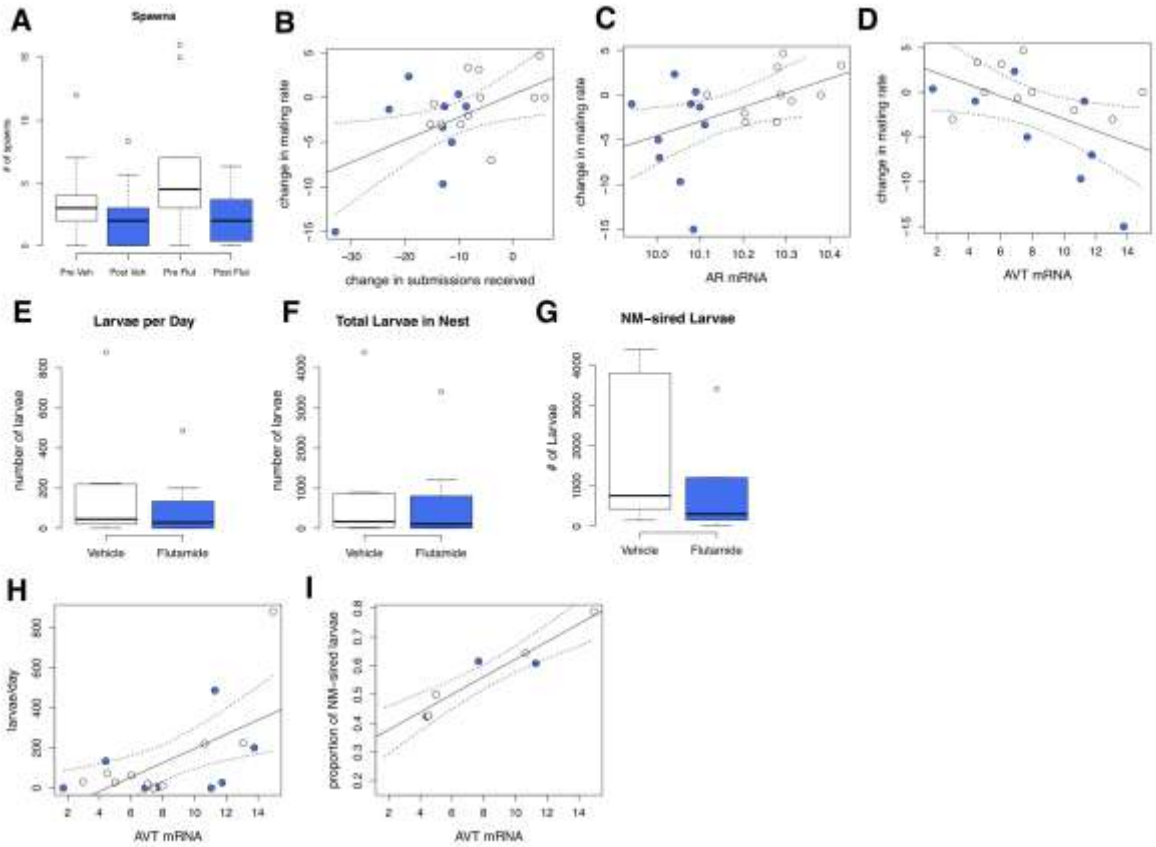

Figure 4