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Electrocommunication behaviour and non invasively-measured androgen changes following induced seasonal breeding in the weakly electric fish, *Apteronotus leptorhynchus*

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ABSTRACT

Androgens are known to be involved in reproductive behaviours including courtship and aggression. According to the Challenge Hypothesis, androgen activity upregulates male reproductive behaviour seasonally and also modulates short term adaptation of these behaviours in response to social context. In the weakly electric fish, *Apteronotus leptorhynchus*, 11-ketotestosterone (11-KT) has been previously implicated in the regulation of electrocommunication behaviours that are believed to have roles in both aggression and courtship. Changes in male 11-KT levels were quantified using a non-invasive measurement technique alongside changes in electrocommunication behaviour following environmental cues that simulated the onset of the breeding season. Males showed an increase in mean electric organ discharge frequency (EODf), which is consistent with earlier results showing a female preference for high EODf. A subset of males with high initial EODfs showed increases in both 11-KT and EODf, which provides support for an EODf-based dominance hierarchy in this species. Males housed in social conditions and exposed to breeding conditioning also showed higher overall electric organ discharge frequencies and 11-KT compared to males housed in isolation. Evidence is presented that another type of electrocommunication signal previously implicated in courtship may also serve as an inter-male signal of submission. Our results are consistent with earlier observations that electrocommunication signals produced during inter-male aggression serve in deterring attacks, and their pattern of production further suggested the formation of a dominance hierarchy.

Introduction

Androgen hormones are involved in regulating male reproduction in vertebrates by promoting the development of secondary sexual characteristics and by modulating reproductive behaviours such as aggression and courtship (for reviews, see Nelson, 2010; Trainor et al., 2009). Seasonal reproduction is facilitated by increasing androgen concentrations to a breeding-season level in response to seasonal cues. This is commonly observed in mammals (for a review see Nelson et al., 1990), birds (e.g., Wingfield et al., 1990), fish (Borg, 1994; Chang and Yueh, 1990; Cornish, 1998; Dahle et al., 2003; Mayer et al., 1990; Sebire et al., 2007) as well as reptiles (e.g., Tokarz et al., 1998). In addition to this seasonal variation in androgen levels, shorter-term modulations can occur as described by the Challenge Hypothesis according to which male androgen-driven behaviours are adapted to shorter-term social contexts (e.g. aggressive fights, establishment of dominance hierarchies, and cues from females; Oliveira et al., 2002, 2009; Wingfield et al., 1990, 2001). For example, the 'winner-effect,' in which winners of aggressive fights or territorial disputes show an increase in androgen levels, is observed in many vertebrate species (e.g., Greenberg and Crews, 1990; Oliveira et al., 1996, 2009; Oyegbile and Marler, 2005), and is a mechanism by which dominance hierarchies can be established and maintained among males (Dugatkin, 1997; Hsu et al., 2006). Courtship behaviour is also driven by androgens (e.g., Arnold, 1975; Ball et al., 2002; Hews et al., 1994; Páll et al., 2002), and courtship cues from females will regulate androgens (e.g., Borges et al., 1998; Oliveira et al., 2002) creating a similar bi-directional relationship between androgens and reproductive behaviour.

The primary androgen of interest in most fish is 11-ketotestosterone (11-KT) (for a review, see Kime, 1993). The link between 11-KT and behaviour has been well studied in fish at the level of seasonal modulations (e.g., Cornish, 1998; Sebire et al., 2007) and in the acute bi-directional responses to, and regulation of, social interactions, aggression, and courtship (e.g., Hirschenhauser and Oliveira, 2006; Hirschenhauser et al., 2008; Oliveira et al., 2002). Weakly electric fish have been shown to be

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a valuable system in which to investigate the links between behaviour and 11-KT and other androgens (for a review, see Stoddard et al., 2006). Weakly electric fish are widely studied because of the ease with which their electrocommunication behaviour can be recorded and analysed; the behaviour consists of the generation of electric signals that can be easily captured by electrodes in the water and quantified (Moller, 1995). This combined with the solid neuroethological understanding of the electroreception system (Bullock et al., 2005) makes electric fish an ideal model organism for studying communication and how it is controlled. However, there are still large gaps in our knowledge of the natural interactions between steroid hormones and behaviour. Here we ask how androgen levels and androgen-linked behaviours change naturally within the reproductive cycle of the gymnotiform weakly electric fish, *Apteronotus leptorhynchus*, or brown ghost knifefish.

Apteronotus leptorhynchus uses a specialised electric organ in its tail to generate a continuous quasi-sinusoidal Electric Organ Discharge (EOD). The frequency of the EOD (EODf) is sexually dimorphic such that males discharge between 850 and 1100 Hz and females between 650 and 850 Hz (Dunlap et al., 1998). Within these ranges, individuals of both sexes maintain a consistent unique frequency (Moortgat et al., 1998). Females have been found to prefer males based on high EODf (O. Bargelletti, J. Gogarten, R. Krahe, personal communication). High EODf is believed to be a good indicator of male quality for several reasons: EODf is correlated with body size (Dunlap, 2002; Triefenbach and Zakon, 2003), males form a dominance hierarchy based on EODf when competing for limited resources (Dunlap and Oliveri, 2002), and having a high EODf may be energetically costly for weakly electric fish in general (Salazar and Stoddard, 2008). Male *A. leptorhynchus* also produce discrete electrocommunication signals called chirps that are believed to be involved in courting females and inter-male aggression (for a review, see Zakon et al., 2002). Chirps are brief increases in EODf coupled with decreases in EOD amplitude, and different stereotyped chirp types have been identified based on frequency excursion and duration (Zupanc et al., 2006). Type 2 chirps are by far the most common chirp type, and males produce them vigorously during physical fights (Hupé and Lewis, 2008; Triefenbach and Zakon, 2008). Type 1 chirps are seen more rarely and are produced by males in response to electrical stimuli that simulate the EOD of conspecific females (Bastian et al., 2001). Both the EODf and chirping behaviour appear to be hormonally regulated in *A. leptorhynchus*; artificially raising plasma 11-KT levels dramatically raises the EODf (Schaefer and Zakon, 1996), and plasma concentrations of 11-KT are correlated with male type 2 chirp production under certain conditions (Dunlap, 2002). In another species of wild-caught weakly electric fish, 11-KT levels have been correlated with male secondary sexual characteristics including EODf range (Cox Fernandes et al., 2010). This has led to the hypothesis that 11-KT regulates masculinisation of both the EODf (to attract females and establish dominance) and type 2 chirp production (to mediate inter-male aggression; Dunlap, 2002). The goal of this study is to determine if 11-KT levels increase both as a mechanism to initiate reproductive behaviours and following increased social activity in groups of captive *A. leptorhynchus* males that are induced to spawn by environmental cues, and if there are correlated changes in electrocommunication behaviour.

Methods

General fish care

Apteronotus leptorhynchus were obtained from a local tropical fish importer (Below Water, Montreal, Quebec) and housed in individual and group aquaria in a temperature controlled room on a 12 h:12 h light:dark cycle. Water conditions were maintained within the following ranges: temperature 27–29 °C, conductivity 200–500 μS (a conductivity range that simulates the dry season) and pH 6.5-7.5. Shelters were provided in the form of artificial plants and sections of PVC tube. Fish were fed live black worms or frozen blood worms twice weekly. Sex was identified based on morphology and EODf (males>850 Hz, females<850 Hz) and was confirmed by inspecting the gonads post-mortem. All EODf values reported here are corrected to 27 °C (Q_{10} = 1.62, Dunlap et al., 2000). Fish were habituated to the water sample collection protocol to minimise the stress response (Wong et al., 2008) before collecting experimental samples (detailed description below). *A. leptorhynchus* is nocturnal, and all manipulations described here were performed in the dark and during the animals' subjective night. All animal experimental protocols were approved by the animal care committee of McGill University (Animal Use Protocol #5408).

Validation of holding water measurements

Recently, measurement of hormones in fish has been achieved via the collection of water-borne steroids that are released across the gills into the water (for a review, see Scott et al., 2008), a method that is particularly useful in small fish, in which repeated drawing of blood would be difficult and/or stressful. Thus far, this technique has been validated and used to measure changes in 11-KT during breeding and changing social contexts in three freshwater species (*Neolamprologus pulcher*: Bender et al., 2006; *Gasterosteus aculeatus*: Sebire et al., 2007; *Astatotilapia burtoni*: Kidd et al., 2010). In order to validate the use of this technique in a new species, a correlation between holding water and plasma samples of a given hormone must be demonstrated. Holding water samples and plasma samples were collected from 14 male *A. leptorhynchus*. Eight individually housed males were habituated to the sampling protocol once daily over 3 days and then water and plasma samples were collected. Samples were collected from an additional 6 males taken from a group tank used in the conductivity drop experiment (see below) 1 week after the conclusion of that experiment. Fish from a variety of holding conditions were used to sample a large physiological range of hormone release rates and plasma levels, in order to maximise the effective range of the validation. Holding water and plasma samples collected from each fish were assayed and extracted using the protocols outlined below.

Experimental setup

Previous studies have determined that groups of *A. leptorhynchus* will breed following a conductivity drop that simulates the onset of the rainy season (Kirschbaum and Schugardt, 2002; O. Bargelletti, J. Gogarten, R. Krahe, personal communication). Three groups of 9 *A. leptorhynchus*, (6 males and 3 females per group) were housed in large aquaria (~115 L) for a total of 1 month. During a 2-week acclimation period, water conductivity was maintained at 500–600 μS and the fish were habituated to the water sampling protocol (see below) by collecting water samples regularly (every 3 days) from each individual. After this period, holding water samples were collected from each fish, followed immediately by a chirp assay and a measurement of mass and EODf. These measurements were done over 3 days between 1 and 5 h after the onset of darkness. Conductivity was then dropped to 50 μS over 5 days (~100 μS drop per day) by replacing tank water with fresh deionised water. Conductivity was held at 50 μS for 1 week and then holding water, chirp response, EODf and mass measurements were repeated again over 3 days between 1 and 5 h after lights out.

Chirp response assay

Chirping response to electrical stimuli was measured for each individual fish using a protocol similar to that used in previous studies (e.g., Bastian et al., 2001; Dulka and Maler, 1994). Chirp assays were performed in a 50×32×25 cm tank filled with 15.6 L of water from the fish's home aquarium. During the chirp assay, fish were confined

in a 'chirp chamber', a PVC shelter tube with the sides cut out and replaced with 1×1 mm plastic netting, permitting the fish to swim freely but not turn around within the chirp chamber. Silver tip recording electrodes were placed at the head and tail of the fish. Stimulating electrodes were placed perpendicular to the axis of the fish's body 8 cm apart and 6 cm away from the fish based on the arrangement recommended in Kelly et al. (2008). Stimulus intensity was calibrated to be 1 mV/cm at the skin surface of the fish.

Each stimulus was a sine wave a certain frequency difference (Df) above or below the fish's own EODf (measured at the beginning of each stimulus presentation). These sine waves mimic the presence of a conspecific fish with an EODf at a certain frequency above or below the fish's own EODf. As in Bastian et al. (2001), large negative Dfs are expected to be interpreted by males as the EODs of conspecific females. Similarly, small Dfs in either direction are expected to be interpreted as conspecific males. Large positive Dfs were included as a control and are expected to be interpreted by females or lower EODf males as higher EODf males. The Dfs used in this experiment were ±200, 100, 50, 20 and 5 Hz. Stimuli were generated and delivered using a desktop computer running MATLAB (The Mathworks, Natick, MA; sampling rate 40 kHz) via a digital/analogue interface (NI PCI-6259M Series Multifunction DAQ; National Instruments Inc., Austin, TX), and stimulus isolator (Model 2200; A-M Systems, Carlsborg, WA). Recordings were made digitally onto a desktop computer via the same digital/analogue interface and an amplifier (A-M Systems 1700) using MATLAB (sampling rate 40 kHz). Each fish was placed in the chirp assay setup for a 15-min acclimation period and was then presented with 10 randomly ordered stimuli. Each stimulus was presented for 2 min with a 6 min break between stimuli. The number of type 1 and type 2 chirps produced during the stimulus presentation was counted semi-automatically using a custom-written MATLAB script that identifies chirps based on duration and frequency excursion from the baseline EODf. Type 1 and type 2 chirps were distinguished based on a 200 Hz cutoff (Type 2<200 Hz<Type 1), which is well established as a reliable distinguishing factor (Bastian et al., 2001; Fugère and Krahe, 2010).

Water sample collection

Sampling of holding water was done on individual fish by placing them in a 5 L beaker filled with 2 L of clean aquarium water for 30 min. The sampling water was deionised carbon-filtered water conductivity-matched to each fish's home aquarium using aquarium salts (per 1 L of deionised water: 10 g $MgSO_4$ * 7 H_2O; 4 g KCl; 1.1 g NaH_2PO_4 * H_2O; 63 g $CaSO_4$ * 2 H_2O) and kept at 27 °C using a water bath. After 30 min, the fish was returned to its home aquarium and water samples were transferred to polypropylene bottles and frozen at −20 °C until extraction. Glassware was rinsed with ethanol and carbon-filtered de-ionised water before use. Water-borne concentrations measured in this way are reported as a release rate corrected for gill surface area by dividing by body mass, or picograms released/grams of fish mass/hour; pg/g/h (Bender et al., 2006; Scott et al., 2008).

Plasma sample collection

Plasma samples were collected via caudal severance of anaesthetised fish. Immediately following the final holding water sample collection, the fish was transferred to an overdose of buffered MS-222 (500 ppm) solution for 1 min and then blood was collected for 3 min using heparanised glass capillaries (#22-260-950; Fisher Scientific, Hampton, NH). Blood was transferred to a 1.5 ml microtube and spun at 13000 rpm for 2 min, and then plasma was separated and frozen at −80 °C.

Hormone extraction

Solid phase extraction (SPE) was used to extract 11-KT from holding water samples. Water samples were thawed overnight to room temperature and then filtered through filter paper (#09-760F; Fisher) to remove particulate material. Extraction of steroid hormones from water was performed using Sep-Pak C-18 Solid Phase Extraction Cartridges (#WAT020515; Waters Corporation, Milford, MA). The SPE cartridges were primed by flushing them with 4 ml 100% methanol followed by 4 ml Millipore-filtered water (Millipore Corporation, Billerica, MA) using a 12-sample vacuum manifold (Alltech, Lexington, KY). A 250 ml aliquot was then taken from each water sample and drawn through Saint-Gobain Tygon tubing (#AER00003) and into the primed SPE cartridge. Steroid hormones were immediately eluted from the cartridges into 13 mm×100 mm glass screw-top vials using two 2 ml aliquots of ethyl acetate dispensed from a glass pipette directly into the SPE cartridge and drawn through by the manifold. The 4 ml eluted solvent was then dried under a nitrogen stream using an Evap-o-rac drying rack (# EW-01610-15; Cole-Parmer Canada Inc, Montreal, Quebec) submerged in a 35 °C water bath. The dried pellet was frozen at −20 °C for a maximum of 7 days. To assess the recovery rate of the extraction protocol we used, 'spikes' were prepared by adding known amounts of hormone standard provided with the enzyme-immunoassay kits (EIA, see below) to 250 ml aliquots of Millipore filtered water. By comparing the amount of hormone reported from the assay of these samples to the known amount originally 'spiked' into water, the recovery rate of the extraction can be determined. Two such spikes were then extracted alongside samples and assayed in replicates on 4 plates to assess extraction recovery rate and inter-assay variation. Two samples of clean aquarium water were also extracted to ensure the absence of steroid hormones.

Steroid hormones were extracted from plasma using an organic phase extraction based on a protocol provided by Anya Goldina (personal correspondence). The plasma was thawed to room temperature and a 20 µl aliquot was then diluted to 250 µl in Millipore-filtered water. Each sample was extracted 3 times using a 1 ml aliquot of a 9:1 mixture of ethyl acetate and hexane (Sigma-Aldrich 178918). The water phase was frozen by placing the test tube in dry ice to facilitate collecting the organic phase. The samples were then dried under a nitrogen stream and the dried pellet was frozen at −20 °C for, at most, 1 week until being resuspended in EIA buffer. To assess recovery rate, 4 plasma spikes were prepared by combining plasma from 3 fish samples into a pooled sample and spiking this pool with a known quantity of hormone standard provided with the EIA kits.

Enzyme immunoassay

Water-borne and plasma 11-KT was assayed using EIA kits from Cayman Chemical (Ann Arbor, Michigan, 11-KT #582751). The IC_{50} of these kits is ~5 pg/ml and the detection limit is ~1.3 pg/ml. The dried samples were resuspended in 5% ethanol and the provided kit buffer. Different concentration factors (from 30× to 50× for water samples) and dilution factors (from 1:50 to 1:600 for plasma samples) were achieved relative to the original sample depending on the volume of buffer and ethanol the samples were resuspended in. Within 48 h of resuspension, EIA kits were run according to manufacturer instructions. Development time to a B_0 between 0.7 and 0.9 was always 15–20 min. In all cases, samples were run in triplicate for at least 2 dilution factors. Inter-assay variability was measured by assaying two spiked water samples extracted with the above protocol on four plates and by running certain fish water samples on multiple plates.

Statistical analysis

Data was organised using Microsoft Excel and statistical procedures were performed in SPSS (IBM, Somers, NY). EIA kit results were

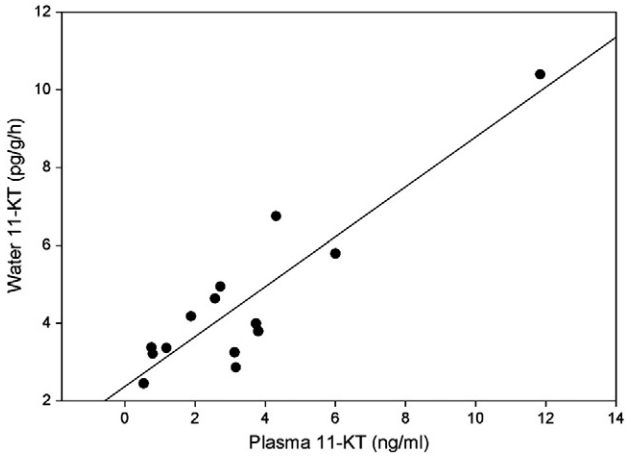

Fig. 1. Positive correlation (Pearson's correlation, $p=0.0001$, $r=0.903$ $n=14$) between measurements of 11-KT made from plasma (ng/ml) and holding water (pg/g/h). Fit line equation is $y=2.37x+0.642$, $r^2=0.816$ where $y=$ water 11-KT (pg/g/h) and $x=$ plasma 11-KT (ng/ml).

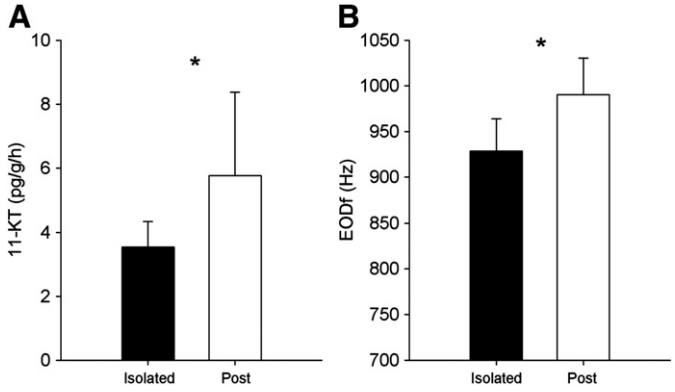

Fig. 2. Comparison of mean values for 11-KT (A), EODf (B) between males housed individually with no environmental changes (filled bars) and males housed in social groups with environmental cues that trigger breeding (open bars). Males housed in social groups with breeding conditioning had significantly higher EODfs and 11-KT ($p<0.05$, marked with a *).

analysed using an Excel spreadsheet provided by the kit manufacturer. Correlations were determined using Pearson's coefficient of correlation, and when appropriate using Spearman's coefficient. Pre- and post-conductivity drop data were analysed using a repeated measure MANOVA.

Results

Extraction and assay controls

Average recovery rates for hormone spikes were 102.4% for SPE extracted samples ($n=2$) and 68.8% for samples extracted from plasma ($n=4$). Coefficients of variation (CV) of between-spike replicates were low (< 10% between all spikes). Inter-assay controls between plates showed a similarly low CV of 9.1% and intra-assay controls showed a CV of 2.09%. All samples were assayed in two dilutions per plate and in some cases more than two dilutions were used in order to capture high physiological variability. Two concentration factors were needed to capture the large physiological range of 11-KT values seen; samples were assayed at 50× concentration factor in most cases but in a few cases where 11-KT release rate was high ($n=3$), a 30× concentration was used. CV between 30× and 50× concentrations was always <20%. High variability in plasma 11-KT levels between individuals required 3 dilution factors, 1:50, 1:200 and 1:600 to assay all fish; the inter-dilution CV for all 3 dilutions was 17%. Levels of 11-KT measured from plasma with EIA were similar to those measured with radioimmunoassay in the same species (Dunlap et al., 2002) and with EIA in other species (Salazar and Stoddard, 2009).

Validation

There was a significant positive linear correlation between plasma and water-borne 11-KT concentrations in males (Pearson's correlation, $p=0.0001$, $r=0.903$ $n=14$) (Fig. 1). Plasma concentrations of 11-KT in males ranged from 540 pg/ml to 11.8 ng/ml. Excretion rate of 11-KT in males ranged from 2.27 pg/g/h to 10.39 pg/g/h. Males sampled following the conductivity drop experiment had significantly higher EODf (MANOVA, $df=1,13$, $F=10.675$, $p=0.006$) and water 11-KT ($df=1,13$, $F=6.562$, $p=0.024$) than males who were housed in isolation (Fig. 2).

Effect of conductivity drop

Mean values for male EODf, mass, 11-KT excretion and total type 1 and type 2 chirp rate in response to all Dfs were compared pre- and post-conductivity drop using a repeated measures MANOVA (Table 1). There was a significant increase in EODf (RM MANOVA, $df=1$, 16, $F=44.435$, $p=0.0001$, $n=17$ males) by an average of 44.7 Hz ± 27.64 (mean ± standard deviation). The range of 11-KT excretion values measured across all males strongly varied from below the threshold of detection to 29.97 pg/g/h. There was no significant change overall in 11-KT excretion from pre- to post-conductivity drop. Looking across all males,

Table 1
Mean and standard deviation values for major measurements. EODf is given in Hz adjusted to 27 °C (Dunlap et al., 2000). Type 1 and Type 2 chirps give the total number of chirps produced to all 10 Dfs. *p* Values are from repeated measures MANOVA within-subjects effects performed for each category of male (all, High EODf or Low EODf). Comparison *p* values of High EODf and Low EODf are from between-subjects effects measured in the all-males MANOVA with High EODf or Low EODf as a block. Significant *p* values ($p<0.05$) are marked with a *.

		EODf (Hz)			Mass (g)			11-KT (pg/g/h)			Type 1 chirps			Type 2 Chirps		
		Pre	Post		Pre	Post		Pre	Post		Pre	Post		Pre	Post	
All males	Mean	922.0	966.7	$p=0.0001^*$	15.7	16.0	$p=0.330$	6.52	9.78	$p=0.166$	38.8	35.5	$p=0.881$	577.0	572.6	$p=0.865$
$n=17$	Standard deviation	35.7	45.1		6.4	7.6		7.65	6.30		40.0	53.0		258.7	282.0	
High EODf males	Mean	938.5	994.2	$p=0.0001^*$	18.4	18.9	$p=0.376$	5.04	11.7	$0=0.009^*$	36.6	17.9	$p=0.244$	552.4	566.0	$p=0.849$
$n=9$	Standard deviation	31.3	27.6		6.8	7.6		5.34	7.62		39.4	24.9		268.0	310.1	
Low EODf males	Mean	903.5	935.7	$p=0.029^*$	12.3	12.9	$p=0.583$	8.19	7.64	$p=0.884$	41.2	55.2	$p=0.669$	604.8	580.1	$p=0.832$
$n=8$	Standard deviation	32.5	41.5		5.5	7.6		9.75	3.79		43.3	69.8		263.1	267.8	
High EODf vs Low EODf males				$p=0.007^*$			$p=0.070$			$p=0.870$			$p=0.196$			$p=0.783$

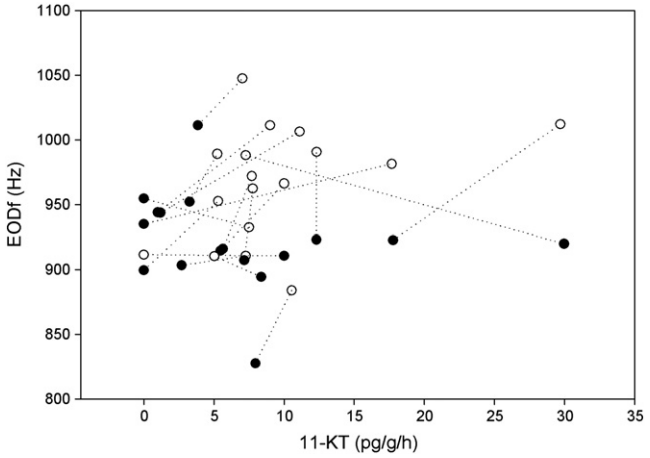

Fig. 3. No correlation between 11-KT and EODf in all males pre- (filled) or post- (open) conductivity drop.

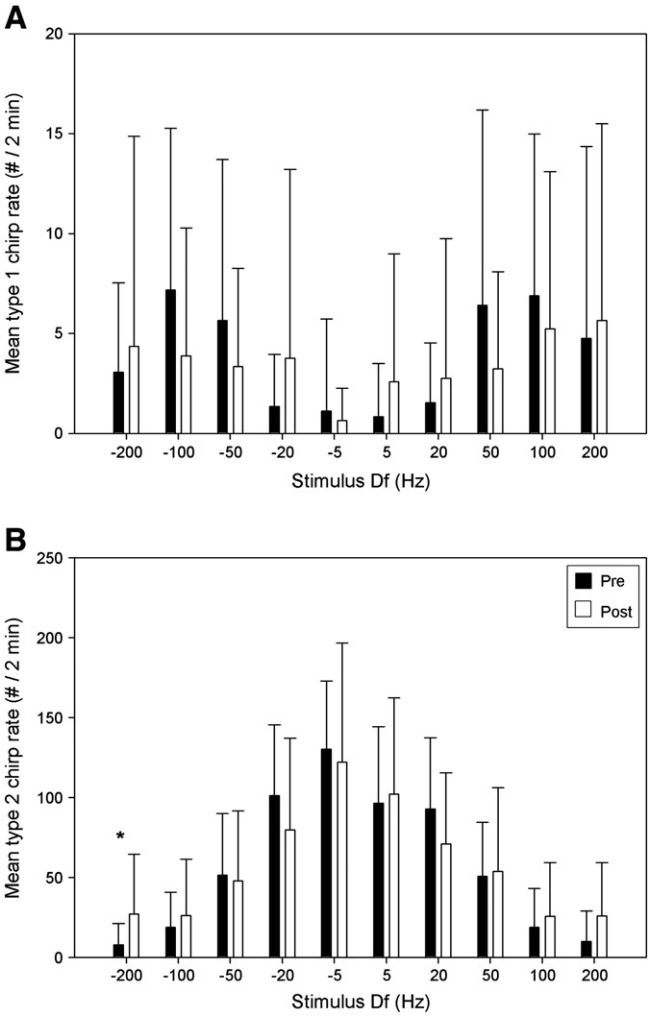

Fig. 4. Mean rate of production of type 1 (A) and type 2 (B) chirps across all males ($n=17$) in response to all Dfs pre- (filled bars) and post- (open bars) conductivity drop. Error bars are standard deviations. Significant difference ($p<0.05$) between pre and post measurement was seen only in the production of type 2 chirps to a Df of −200 Hz and is indicated with a *. Type 1 chirp production was tuned mostly to large Dfs (±50 Hz to 200 Hz), whereas type 2 chirp production was tuned mostly to small Dfs (−20 Hz to +20 Hz).

EODf and 11-KT were not correlated pre- or post-conductivity drop (Fig. 3).

Male type 1 and type 2 chirp response showed the same tuning to different Dfs demonstrated previously (Bastian et al., 2001; Fig. 4). There was no significant change in mean total type 1 or type 2 chirp rate in response to all Dfs (Table 1) following the conductivity drop. Mean type 2 chirp rate in response to a Df of −200 Hz increased significantly ($p=0.005$, $n=17$, marked with an asterisk in Fig. 4).

Type 2 chirp rate in response to a Df of −5 Hz was negatively correlated with EODf post-conductivity drop ($p=0.039$, $r=0.504$, $n=17$; Pearson's). There were no other significant correlations between EODf, 11-KT excretion, mass, and total type 1 and type 2 chirp responses across all males. There were also no significant differences between the 3 tank replicates ($p=0.139$, $df=2$, between-subjects factor in RM MANOVA).

High EODf males

Male *A. leptorhynchus* and related species are believed to form dominance hierarchies based on EOD frequency (Dunlap and Oliveri, 2002; Fugère et al., 2011; Hagedorn and Heiligenberg, 1985). Based on this hypothesis, we looked at the subsample of males comprised of the 3 highest EODf males from each replicate tank (the top half). As expected, this group of males differed significantly from the remaining low EODf males based on EODf ($p=0.007$ $df=1,15$, between-subjects factor in RM MANOVA, see Table 1). Among high EODf males, the same significant increase in EODf post-conductivity drop was observed ($p=0.0001$, $df=1,8$, RM MANOVA, Table 1). Contrary to the result for the whole group, but similar to what was found in the males used for the validation of the holding water method, a significant increase in 11-KT excretion was also observed ($p=0.008$, $df=1,8$, RM MANOVA). Each individual showed an increase in both EODf and 11-KT, although EODf and 11-KT were not correlated pre- or post-conductivity drop. Individual changes (post value minus pre value) in EODf and 11-KT values show a non-significant trend (Fig. 5). A significant positive correlation is found after removal of an outlier ($p=0.029$, $r=0.795$, $n=8$). To further assess this possible relationship in high EODf males, we also performed a linear mixed model analysis using individual fish as a random factor, EODf as a function of 11-KT and time (pre- or post-conductivity drop) as a categorical variable. This analysis did not yield a significant correlation between 11-KT and EODf either, but it did show a significant interaction of 11-KT and time on EODf ($F=7.26$, $p=0.033$) with the pre-conductivity-drop slope being smaller than the slope measured after the conductivity-drop. A significant positive correlation between EODf

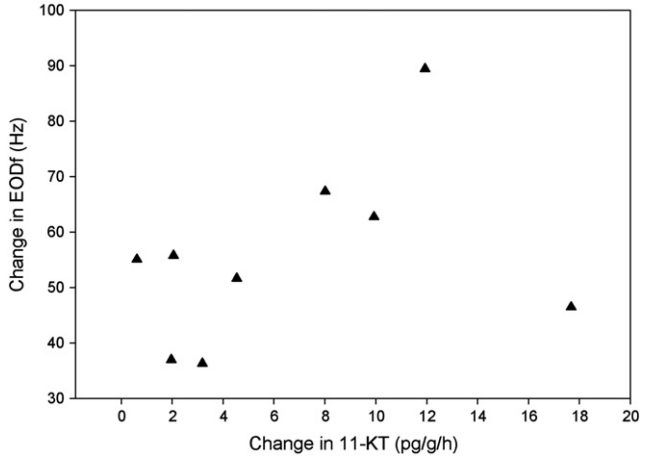

Fig. 5. Change in 11-KT and in EODf in high EODf males (post- minus pre-conductivity drop). Removal of outlier (bottom right) yields a significant positive correlation ($p=0.029$, $r=0.794$, $n=8$).

and mass was also observed in high EODf males (pre: $p=0.012$, $r=0.786$, $n=9$; post: $p=0.008$, $r=0.810$, $n=9$).

Pre-conductivity drop, total type 1 chirp production was negatively correlated with 11-KT in high EODf males (Spearman's, $p=0.004$, $r_s=0.845$, $n=9$) in what appears to be a curvilinear relationship (Fig. 6A). This correlation was not found after the conductivity drop. Post conductivity drop, total type 2 chirp production was similarly negatively correlated with EODf; specifically, type 2 chirp production to Dfs of −50 Hz, −20 Hz, −5 Hz and +5 Hz were all negatively correlated with EODf (Df = −50 Hz: $p=0.001$, $r_s=0.900$; Df = −20 Hz: $p=0.004$, $r_s=0.850$; Df = −5 Hz: $p=0.025$, $r_s=0.733$; Df = +5 Hz: $p=0.010$, $r_s=0.800$; $n=9$) (Fig. 6B).

Positive correlations between EODf and type 1 chirp production were observed for certain Dfs, but they appeared to be driven by individual males (data not shown). Specifically, one high EODf male drove positive correlations between EODf and type 1 chirp production to certain Dfs (−200 Hz, −20 Hz, +20 Hz) pre-conductivity drop and to mainly small Dfs (−20 Hz, −5 Hz, +5 Hz) post-conductivity drop. Similarly, the male with the highest 11-KT measured drove positive correlations between 11-KT and type 1 chirp production to certain Dfs (−200 Hz, +20 Hz) post conductivity drop.

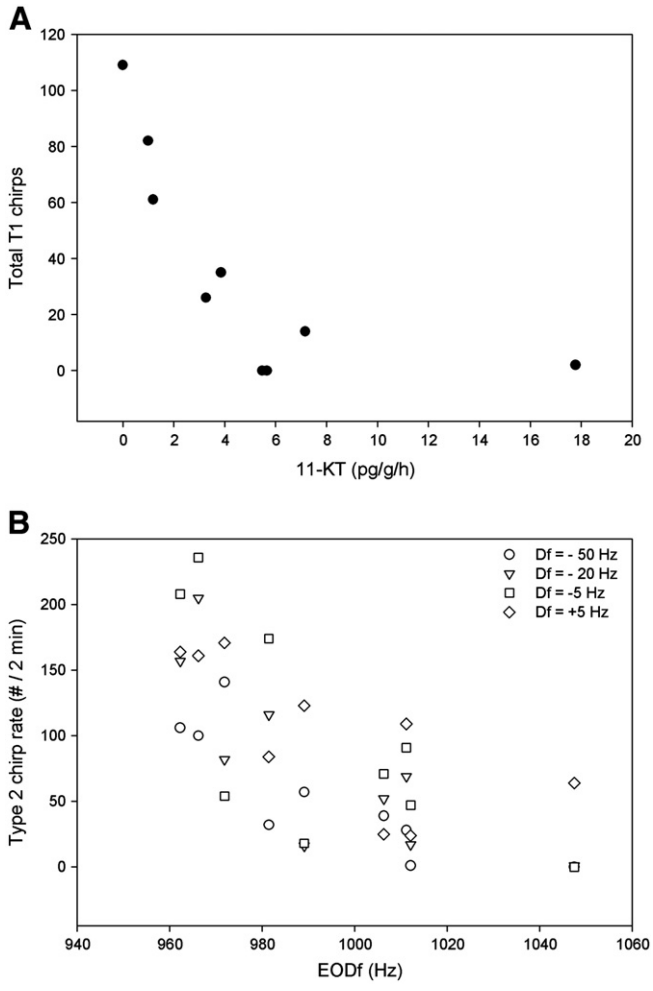

Fig. 6. Relationships between chirp production and 11-KT and EODf in high EODf males ($n=9$). A: Negative relationship between total type 1 chirp production to all Dfs and 11-KT pre-conductivity drop (Spearman's, $r_s=0.845$, $p=0.004$, $n=9$). B: Negative relationships between type 2 chirp rate to several small Dfs and EODf post-conductivity drop ($n=9$, Df = −50 Hz: $r_s=0.900$, $p=0.001$, Df = −20 Hz: $r_s=0.850$, $p=0.004$, Df = −5 Hz: $r_s=0.733$, $p=0.025$, Df = +5 Hz: $r_s=0.800$, $p=0.010$).

Low EODf males

Low EODf males also showed a significant increase in EODf ($p=0.029$, df = 1,7, RM MANOVA), but no significant changes in mass, 11-KT or type 1 and type 2 chirping to any Dfs were observed post-conductivity drop. One low EODf male died during the conductivity drop and values from this fish were omitted.

Among low EODf males, there was a significant correlation between EODf and mass only pre-conductivity drop ($p=0.001$, $r=0.935$, $n=8$; data not shown). Pre-conductivity drop, a single male showed extremely high type 1 chirp rates to Dfs of −200 Hz, +100 Hz and +200 Hz. Post-conductivity drop, 11-KT was negatively correlated with type 2 chirp rate to 3 large Dfs, −200 Hz, +100 Hz and +200 Hz (Pearson's, Df = −200 Hz: $p=0.044$, $r=0.720$; Df = +100 Hz: $p=0.011$, $r=0.829$; Df = +200 Hz: $p=0.011$, $r=0.830$; $n=8$) (Fig. 7).

Discussion

Validation

When the technique of water-borne hormone measurements is to be applied to a new hormone or a new species, a validation with respect to plasma levels is required (Scott et al., 2008). Here we have demonstrated a strong correlation between the release rate into holding water and plasma concentration of 11-KT in *A. leptorhynchus*, allowing this technique to be applied with confidence both in this study and in future ones. In this validation, we used a large size range of mature males from a variety of holding conditions to maximise the range within which this validation can be applied. The correlation between plasma concentrations and release rate of 11-KT found here is strong but it should be applied with caution to females or fish beyond the size and 11-KT range used here (Scott et al., 2008).

EOD frequency and 11-ketotestosterone

This study is the first demonstration that male *A. leptorhynchus* increase their EODf following a seasonal cue associated with the onset of the breeding season. A previous study of *A. leptorhynchus* using very high female:male sex ratios (up to 13:2) did not find this change in EODf (O. Bargelletti, J. Gogarten, R. Krahe, personal communication) and a study of wild-caught *Sternopygus macrurus* did not note any difference in male EODf between different seasons of collection (Zakon et al., 1991). Previously, a change in EODf was observed in males under similar breeding conditions in *Eigenmannia virescens*, another wave-type gymnotiform (Westby and Kirschbaum, 1981).

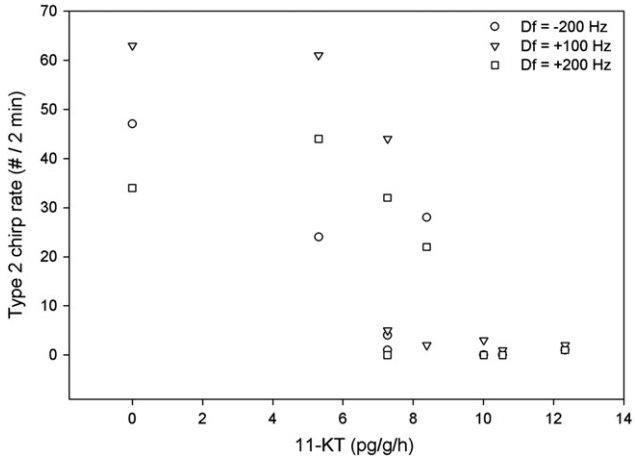

Fig. 7. Negative correlations between type 2 chirp production to large Dfs and 11-KT post-conductivity drop in low EODf males (Pearson's, $n=8$, Df = −200 Hz: $r=0.720$, $p=0.044$, Df = +100 Hz: $r=0.829$, $p=0.011$, Df = +200 Hz: $r=0.830$, $p=0.011$).

This study is also the first to show that male *A. leptorhynchus* housed in social groups have higher EODfs than males housed in isolation. This suggests that males increase their EODf when in a social context; high EODf may be useful to attract females (O. Bargelletti, J. Gogarten, R. Krahe, personal communication) or display dominance over other males (Dunlap and Oliveri, 2002; Fugère et al., 2011). High EODf males showed an increase in EODf following a conductivity drop, further strengthening the idea that high EODf is important when groups of fish are reproductively active.

Males housed in social groups also had higher 11-KT levels than those housed in isolation, and high EODf males all showed an increased in 11-KT levels. However, we found no direct correlation between 11-KT and EODf. The 11-KT increase seen in high EODf males, which are presumed to be the dominant ones, and the absence of an increase in low EODf males, may be indicative of increased aggressive interactions with other males or social interaction with females. We found a positive trend between the change in EODf and 11-KT in these males, and similar coupled increases in reproductive behaviour and 11-KT levels are known to occur in other fish species following social interactions (Borges et al., 1998; Oliveira, 2009), especially in dominant males (e.g., Oliveira et al., 1996, 2009). Previous studies using 11-KT implants (Schaefer and Zakon, 1996) and limited social contact (Dunlap et al., 2002) have shown links between 11-KT and EODf, but it appears that in larger social groups, factors besides 11-KT must contribute to regulating the EODf. Indeed, we have shown that 11-KT levels and EODf both differ between samples of males depending on the social conditions in which they are housed.

Chirping

Overall, type 1 and type 2 chirp production showed the same tuning observed previously, with type 1 chirps produced strongly in response to large negative, or female-like Dfs and type 2 chirps produced mainly in response to small, or male-like Dfs (Bastian et al., 2001). Previously, type 2 chirps were identified as strictly aggressive signals (Bastian et al., 2001; Maler and Ellis, 1987; Triefenbach and Zakon, 2008) and their production was positively correlated with 11-KT under certain conditions only (Dunlap, 2002; Dunlap et al., 2002; these studies are assumed to refer to the more common type 2 chirp since the authors did not distinguish between chirp types). In another study of a closely related apteronotid, no relationship between chirping and 11-KT was observed (Ho et al., 2010). However, fine temporal analysis of type 2 chirp production during aggressive interactions between males reveals that they serve to deter aggressive attacks (Hupé and Lewis, 2008). Additionally, rather than seeing a positive relationship between 11-KT and type 2 chirp rate (which would be expected if these were strictly aggressive signals) we observed no such relationship among high EODf males and a negative correlation among low EODf males in certain situations. Therefore, type 2 chirps do not appear to be dominant or threatening signals and may even have a submissive character in deterring attacks. Consistent with this, our results show that males in reproductive context produce these signals at a rate inversely related to the male's (EODf-based) status in the dominance hierarchy when encountering a new potential rival (i.e. a small Df stimulus). Similarly, among the lowest EODf males, those with very low 11-KT levels produced a comparatively large number of type 2 chirps to stimuli that simulate very high EODf males, further suggesting that these chirps are submissive signals and that 11-KT may also play a role in mediating social status following social interactions as discussed earlier.

We did not find evidence supporting the proposed role of type 1 chirps as courtship signals. Type 1 chirping was not seen to increase following the onset of breeding conditions as is seen with courtship signaling in other fish species (e.g. Sebire et al., 2007) and was in fact negatively correlated with 11-KT in high EODf males pre-conductivity drop. It should be noted that tuning of the type 1 chirp response is not sign specific; although *A. leptorhynchus* can sense the positive or negative direction of small Dfs (Heiligenberg, 1991), males produce type 1 chirps at roughly equal rates to large Dfs whether they are positive or negative (Bastian et al., 2001; Engler & Zupanc, 2001) suggesting that they are not able to discriminate between a positive or negative Df past a certain magnitude. Type 1 chirps could therefore in fact also be directed at males with much higher EODf. Given that 11-KT and type 1 chirp production are negatively correlated, type 1 chirps may therefore be submissive signals directed towards males higher up in the social rank, at least outside of the breeding season. Further investigation of the chirping behaviour of males in real interactions with other fish is required to clarify this issue.

Conclusion

This study represents the first attempt to study changes in 11-KT levels as well as EODf and chirping response in reproductively active groups of *A. leptorhynchus*. Following environmental cues which induce spawning, males increase their EOD frequency, presumably to attract females, and among the highest EODf males there is an increase in 11-KT, which is suggestive of increased social interaction and 11-KT's role adapting male behaviour in reproductive contexts. We also found different relationships between EODf and type 1 and type 2 chirping than those found previously using individually housed fish, suggesting possible alternative roles for these electrocommunication signals. These discrepancies highlight the importance of studying reproductively active and socially housed fish when studying the links between hormones and behaviour. Future studies should make use of this system to address the relationship between 11-KT and communication behaviour on short time scales by measuring 11-KT repeatedly from the same individuals as they are exposed to various social situations, such as chirp stimulation, aggressive interactions, or interactions with females.

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