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ARTICLE

Androgen effects on immune gene expression during parental care in bluegill sunfish (*Lepomis macrochirus*)

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Abstract: Reproductive efforts are energetically costly and often result in significant trade-offs with other metabolic processes. In many animals, androgens influence how this energy is allocated during the breeding period. In this study, androgen levels of parental bluegill sunfish (*Lepomis macrochirus* Rafinesque, 1819) were manipulated by implanting males with either 11-ketotestosterone (11-KT) or flutamide (an androgen receptor antagonist) shortly after spawning. Parental behaviours were assessed for 3 days after implantation. Differential brain gene expression between the two groups was examined using a 44 000 microarray gene chip. We found that 11-KT-implanted males exhibited lower levels of expression of genes related to immune function compared with flutamide-implanted males, suggesting that high androgen levels negatively affect the male immune system. These data thus indicate that a significant trade-off may exist between maintaining high levels of androgens and decreased immune function in parental male bluegill.

Key words: immune function, androgens, parental care, gene expression, bluegill sunfish, *Lepomis macrochirus*.

Résumé : Les efforts de reproduction sont coûteux sur le plan énergétique et se traduisent souvent par d'importants compromis en ce qui concerne d'autres processus métaboliques. Chez de nombreux animaux, les androgènes influencent l'affectation de cette énergie durant la saison de reproduction. Dans cette étude, les teneurs d'androgènes de crapets arlequins (*Lepomis macrochirus* Rafinesque, 1819) parentaux ont été manipulées en implantant dans des mâles soit de la 11-cétotestostérone (11-KT) ou de la flutamide (un antagoniste des récepteurs d'androgènes) peu après le frai. Les comportements parentaux ont été évalués pendant 3 jours après l'implantation. Les différences d'expression des gènes du cerveau entre les deux groupes ont été examinées à l'aide d'une puce à gènes 44 000. Nous avons constaté que les mâles dans lesquels avait été implantée de la 11-KT présentaient des niveaux plus faibles d'expression de gènes associés à la fonction immunitaire que les mâles ayant fait l'objet d'une implantation de flutamide, ce qui donne à penser que de fortes teneurs d'androgènes ont un effet négatif sur le système immunitaire des mâles. Ces données indiquent donc qu'il pourrait y avoir un important compromis entre le maintien de fortes teneurs d'androgènes et la réduction de la fonction immunitaire chez les crapets arlequins mâles parentaux. [Traduit par la Rédaction]

Mots-clés : fonction immunitaire, androgènes, soins parentaux, expression génique, crapet arlequin, *Lepomis macrochirus*.

Introduction

Reproduction is one of the most important times in the life of an individual because reproductive success is a main component of fitness. Reproduction is energetically costly, which leads to trade-offs regarding how energy is allocated during this period (Muehlenbein and Bribiescas 2005; Harshman and Zera 2007). Some of these trade-offs may include how much energy to shunt into the general processes associated with reproduction, such as egg and sperm production, over other metabolically costly processes, such as immune defence (Muehlenbein and Bribiescas 2005). Other trade-offs may occur between different aspects of reproduction, such as time spent courting or time performing parental care behaviours (Sargent 1985).

For parental male bluegill sunfish (*Lepomis macrochirus* Rafinesque, 1819), a significant trade-off exists between the time spent performing either nurturing or aggressive parental care behaviours (Rodgers et al. 2013). In this species, parental males are the sole caregivers for offspring after spawning. They are responsible for cleaning, aerating, and removing dead eggs from nests (i.e., nurturing-type behaviours). In addition, they protect developing offspring by chasing and biting potential predators (i.e., aggressive-type behaviours). These two sets of behaviours appear to be governed by hormonal pathways and may be in conflict with one another. Pituitary hormones such as prolactin are thought to influence the performance of some of these behaviours (Blüm and Fiedler 1965; De Ruiter et al. 1986; Kindler et al. 1991; Páll et al. 2004). For example, males implanted with bromocriptine, a dopamine receptor agonist that inhibits prolactin secretion, exhibited more prespawning behaviours and fewer nest defence behaviours (Kindler et al. 1991). The additional time spent in these prespawning behaviours after eggs were deposited also coincided with a decrease in fanning behaviour, resulting in higher egg mortality due to fungal infections.

In addition to pituitary hormones, steroid hormones also appear to influence how much time males invest in reproductive behaviours. A recent study by Rodgers et al. (2013) showed that parental males implanted with 11-ketotestosterone (11-KT), the dominant androgen in most teleosts, perform significantly more aggressive behaviours toward potential predators, whereas males

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implanted with flutamide, an androgen receptor antagonist, performed significantly more nurturing behaviours, including fanning and cleaning. Similarly, associations between parental care and androgens levels have been observed in other centrarchids. Dey et al. (2010) found that smallmouth bass (*Micropterus dolomieu* Lacepède, 1802) parental males treated with cyproterone acetate (an androgen receptor antagonist and androgen synthesis inhibitor) reduced nest defence behaviours when presented with a potential brood predator. Also, the level of aggression toward a brood predator was correlated with testosterone levels in smallmouth bass and individuals recently experiencing simulated nest intrusions had higher levels of testosterone than individuals who had not (O'Connor et al. 2011). These results suggest that sex hormones such as testosterone (T) and 11-KT play a significant role in regulating aggressive-type behaviours.

In addition to regulating parental care behaviours, sex hormones have also been suggested to influence how energy is allocated during reproduction (Muehlenbein and Bribiescas 2005). One proposed mechanism is that higher levels of testosterone (T) cause a shift in energy allocation away from the immune system into reproductive processes (Folstad and Karter 1992). Numerous studies have shown a significant association between increased circulating T levels and immune suppression (Deerenberg et al. 1997; Suzuki et al. 1997; Casto et al. 2001; Pap et al. 2010), although this effect is not universal (see Hasselquist et al. 1999; Saha et al. 2002; Greenman et al. 2005) and these effects can differ among various aspects of the immune system (Ezenwa et al. 2012). For example, a meta-analysis by Roberts et al. (2004) comparing 22 separate studies found that T did negatively affect immune function. However, this effect was lost after controlling for multiple studies on the same species, suggesting that the effect of T on immune function may vary among species. The mechanism by which T may alter immune function has not been fully evaluated in teleost fishes. In humans, lymphocytes, such as B and T cells, contain androgen receptors and when binding T suppress the production of various immune-related compounds (Kanda et al. 1996). Thus, conceivably, as fish enter into the breeding season and androgen levels increase, the immune system may become compromised.

To understand the genetic factors that may be influenced by increased androgen levels in bluegill sunfish, we manipulated androgen signaling in parental males via implantation of 11-KT and flutamide (an androgen receptor antagonist) shortly after spawning. We assessed differential gene expression in the brain using competitive hybridization to a 44 000 microarray gene chip. Brain tissue was specifically chosen because the primary goal for this study was to access whether we could correlate changes in gene expression with hormone manipulation and changes in paternal care behaviours.

Materials and methods

Animal sampling

Between 4 and 19 June 2010, male parental sunfish were captured from Lake Opinicon, Ontario, Canada, 1 day after spawning. We measured total length and body mass of each male and calculated Fulton's condition factor (an estimate of the male's energetic state) using the equation (mass/length^3) × 10^5 (Neff and Cargnelli 2004). Blood was collected from the caudal blood vessel using heparinized syringes and placed on ice until plasma separation. The plasma was then removed and stored at –20 °C until hormone assay.

Hormone implants

After blood collection, males were anesthetized using clove oil and implants containing either flutamide or 11-KT were inserted into the abdominal cavity. Males receiving 11-KT implants received either one (KT-1) or two (KT-2) implants. In total, 17 males received flutamide implants, 20 males received KT-1 implants, and 23 males received KT-2 implants. Full details regarding construction of the hormone implants are provided in Rodgers et al. (2013). In short, 11-KT implants were made of silastic tubing (Konigsburg Instruments, Pasadena, California, USA) and were 7 mm long (1.47 mm inner diameter, 1.96 mm outer diameter) with 1 mm silicone sealant on each end. The 11-KT implants were prepared by dissolving crystalline 11-KT in ethanol and then mixed with castor oil for a final concentration of 8 mg 11-KT/mL oil. Flutamide implants were 8 mm in total length and consisted of crystalline flutamide packed into silastic tubing.

For the next 2 days, individual behaviours were recorded to examine how the hormone implants affected parental care behaviours in the males (full details are outlined in Rodgers et al. 2013). These behaviours were categorized into nurturing behaviours (pectoral fanning, caudal sweeps, and egg removal) and aggressive behaviours towards a potential egg predator (number of bites, opercular flares, and lateral displays). The ratio of nurturing to aggressive behaviours was then calculated by dividing the total number of nurturing behaviours by the total number of aggressive behaviours.

Three days after implantation, males were recaptured. Because of nest abandonment by some males, 9 flutamide, 10 KT-1, and 10 KT-2 males were sampled for further analysis. Length and body mass were again measured, Fulton's condition index calculated, and blood was collected. Males were then euthanized in clove oil and brain samples were immediately removed and stored in RNAlater (Life Technologies, Carlsbad, California, USA).

Hormone analysis

Plasma levels of T and 11-KT were measured using radioimmunoassay (RIA) after chromatographic separation (Magee et al. 2006). Full details of the methods are provided in Rodgers et al. (2013).

Total RNA extraction

Total RNA was extracted using the standard Trizol (Invitrogen, Carlsbad, California, USA) extraction protocol and RNA concentration was determined using a Nanodrop™ 2000 (Thermo Scientific, Wilmington, Delaware, USA). Samples were submitted to the London Genomics Center at the University of Western Ontario and RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, California, USA). Because of the time between tissue sampling and RNA extraction, only a subset of the total brains collected were of high enough quality for gene expression analysis. This included four brains from KT-1 males, two brains from KT-2 males, and four brains from flutamide males. The quality of RNA from the KT-2-treated males was not sufficient for array analysis, thus only KT-1 and flutamide individuals were used in the microarray analysis.

Microarray

Two-color arrays were used to assess differential gene expression between KT-1 males and those implanted with flutamide. We used a 4 × 44 000 Agilent microarray (Agilent, Santa Clara, California, USA) initially developed by EcoArray (Alachula, Florida, USA). This array consists of over 44 000 transcripts derived from smallmouth bass and largemouth bass (*Micropterus salmoides* (Lacepède, 1802)), which like bluegill are members of the teleost family Centrarchidae. After RNA quality was determined, samples were labeled and amplified using Agilent Low Input Quick-Amp Labeling Kits (Agilent, Santa Clara, California, USA).

Three biological replicates from KT-1 and flutamide treatments were competitively hybridized. A technical replicate with dye swap was performed for each of the biological replicate pairs. Samples were hybridized for 19 h at 65 °C. After hybridization, chips were washed and immediately scanned using a GenePix 4300A scanner (Molecular Devices, Sunnyvale, California, USA).

Table 1. Physical, behavioural, and plasma hormone characteristics (mean ± SE) of 11-ketotestosterone-implanted (11-KT) and flutamide-implanted parental male bluegill sunfish (*Lepomis macrochirus*).

	KT-1 (n = 4)	KT-2 (n = 2)	Flutamide (n = 4)	df	F	p
Total length (mm)	198.8±6.4	212.5±5.5	198.8±4.8	2, 7	1.9	0.34
Body mass (g)	149.0±14.4	194.0±8.0	148.8±8.7	2, 7	3.2	0.10
Fulton's condition factor	1.9±0.02	2.03±0.08	1.9±0.06	2, 7	1.8	0.24
11-KT (ng/mL)	16.6±4.3	15.6±4.4	22.5±7.7	2, 7	0.3	0.73
Testosterone (ng/mL)	3.8±1.1	3.9±2.0	4.6±1.3	2, 7	0.1	0.89
Total no. of nurturing behaviours	6.0±2.4	1.0±1.0	38.0±10.0	2, 7	**7.6**	**0.02**
Total no. of aggressive behaviours	24.5±1.9	23.0±3.0	14.8±5.0	2, 7	2.0	0.21
Total no. of behaviours	30.5±1.8	24.0±2.0	52.8±14.2	2, 7	1.9	0.36
Ratio of nurturing to aggressive behaviours	0.27±0.12	0.05±0.05	4.7±2.1	2, 7	3.1	0.11

Note: Values in boldface type are statistically significant at $p < 0.05$. KT-1, males implanted with one 11-KT implant; KT-2, males implanted with two 11-KT implants.

Spot intensity was assessed using GenePix Pro 7 (Molecular Devices, Sunnyvale, California, USA).

Microarray statistical analysis

Background subtraction, normalization, and analysis of differential expression were performed using the linear models for microarray data (LIMMA) package in R (Smyth 2005). Data were background corrected using the "normexp" method with an offset of 25. These data were then normalized using global loess normalization. Spots consisting of the same transcripts were then averaged together. This left 29 191 unique transcripts for statistical analysis. Correlation estimates among technical replicates were calculated for each gene using a linear mixed model estimate and a contrast matrix was used to examine expression levels of KT-1 males compared with flutamide males. Tests of significant differences were performed using an empirical Bayes method. After statistical analysis, transcripts with expression levels lower than negative controls (<6.5) were removed from the data set, leaving a total of 4827 transcripts. A false discovery rate (FDR) correction was applied and genes with $p < 0.1$ after FDR correction were considered to be significant.

Quantitative PCR

Quantitative PCR (qPCR) was performed for the immunoglobulin heavy-chain gene (GenBank accession No. AF327364 from the Mandarin fish, *Siniperca chuatsi* (Basilewsky, 1855)), which showed the highest level of differential expression from the microarray, and for prolactin (Prl) (GenBank accession No. AB219243 from red seabream, *Pagrus major* (Temminch and Schlegel, 1843)), a hormone previously implicated in male parental care in sunfish (Kindler et al. 1991). Because of limited sample availability, only three KT-1 males, two KT-2 males, and three flutamide males were used for verification of gene expression using qPCR. Both KT-2 individuals had RNA of moderate quality with RIN scores of 6.3. Four samples (two KT-1 and two flutamide) had also been used in the microarray analysis. RNA was extracted as previously described. All samples were treated with DNA-*free*™ (Ambion, Carlsbad, California, USA) to remove genomic DNA. Samples were reverse-transcribed using a qScript™ cDNA synthesis kit (Quanta, Gaithersburg, Maryland, USA). Relative gene expression was quantified using SYBR® Green PCR master mix (Applied Biosystems, Carlsbad, California, USA) and normalized against β-actin (GenBank accession No. AB179839 for the Japanese amberjack, *Seriola quinqueradiata* Temminck and Schlegel, 1845), which served as the reference gene. The thermal cycle protocol consisted of an initial denaturing step at 95 °C for 10 min followed by 44 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, then followed by a melt curve.

An ANOVA was used to determine statistical differences in gene expression between all treatments. To examine the relationship between prolactin gene expression and the number of nurturing behaviours performed by parental males, a general linear model with a quasi-Poisson distribution was employed. All statistical analyses were performed with R version 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria; available from http://www.r-project.org/).

Results

Parental male traits

Physical, behavioural, and hormonal data collected after implantation for the parental males used in this study are presented in Table 1. These data are a subset from the larger data set from Rodgers et al. (2013). The total number of aggressive behaviours, the relative number of nurturing behaviours to aggressive behaviours, and the total number of behaviours were not influenced by treatment. However, flutamide males did show significantly more nurturing behaviours compared with KT-1 or KT-2 males. These differences are consistent with what was observed in the complete data set from Rodgers et al. (2013).

Global gene expression

Of the 29 191 transcripts analyzed, 4 transcripts were found to be significantly different between KT-1 and flutamide treatments. These transcripts are associated with two specific genes, immunoglobulin heavy chain and major histocompatability complex (MHC) II alpha. In addition, out of the top 10 transcripts, 5 transcripts are associated with genes involved in immune processes (Table 2). For those that were significantly different, expression levels were lower in KT-1 individuals compared with flutamide males.

Quantitative PCR

Quantitative PCR results were similar to, but did not statistically support, the microarray data, with a possible explanation related to the low power of the analysis due to small sample size. When expression values of immunoglobulin heavy chain were standardized to β-actin, relative expression did not differ significantly among treatments (KT-1: 0.49 ± 0.29, KT-2: 0.04 ± 0.03, flutamide: 0.64 ± 0.22; $F_{[2,7]} = 1.4$, $p = 0.3$). However, the pattern observed was the same as with the array data, with KT-implanted males displaying relatively lower gene expression than flutamide males.

Prolactin gene expression did not differ between treatments (KT-1: 0.81 ± 0.42, KT-2: 0.002 ± 0.002, Flutamide: 0.20 ± 0.19; $F_{[2,7]} = 1.9$, $p = 0.3$). This corresponds to what was observed with the array, in that KT-1 males tended to show slightly higher expression values but not significantly so. A general linear model showed that prolactin mRNA levels were also not significantly associated with the number of nurturing behaviours performed ($Z_{[1,6]} = 0.35$, adjusted $R^2 = -0.04$, $p = 0.97$, $n = 8$).

Discussion

The present study provides support for the idea that 11-KT influences the immune system in parental male bluegill. Microarray data showed that two genes involved in immune function, immunoglobulin heavy chain and MHC II alpha, exhibited significantly

Table 2. Top ten differentially expressed genes in bluegill sunfish (*Lepomis macrochirus*).

Gene transcript	Accession No.	Log_2-fold change in flutamide	Mean expression	Student's *t* test	*p*	Adjusted *p*
Immunoglobulin heavy-chain mRNA (*Siniperca chuatsi*)	**AF327364**	**1.61**	**10.36**	**7.75**	**0.00**	**0.01**
Immunoglobulin heavy-chain mRNA (*Siniperca chuatsi*)	**AY885709**	**1.25**	**8.81**	**7.18**	**0.00**	**0.01**
MHC class II alpha subunit (*Stizostedion vitreum*)	**AY158871**	**0.82**	**7.72**	**5.30**	**0.00**	**0.05**
MHC class II alpha antigen (*Sparus aurata*)	**DQ019401**	**1.59**	**6.54**	**4.96**	**0.00**	**0.08**
Immunoglobulin light chain (*Seriola quinqueradiata*)	AB064322	0.80	8.03	4.78	0.00	0.10
Tetraodon nigroviridis full-length cDNA	CR729406	0.58	8.15	4.77	0.00	0.10
BAC clone RP24-388D8 from chromosome 3 (*Mus musculus*)	AC122490*	0.63	9.50	4.73	0.00	0.10
Anserinase (*Oreochromis niloticus*)	AB179777	0.67	9.72	4.59	0.00	0.13
Sequence from clone RP1-187J11 on chromosome 6q11.1-22.33 Contains the 3′ part of the gene for endoplasmic reticulum associated protein 140 kDa (ERAP140) (*Homo sapiens*)	AL035689*	0.69	6.53	4.54	0.00	0.13
Spermatogenesis associated glutamate (E) rich protein 4b (Speer4b) (*Mus musculus*)	NM_028561*	−0.71	6.76	−4.32	0.00	0.19

Note: Items in boldface type represent significant differences at $p < 0.1$ after a false discovery rate (FDR) correction. An asterisk denotes weak BLAST hit with $p > 0.05$. Log_2-fold changes represent expression values of flutamide-implanted males relative to KT-1-implanted males. Gene transcripts from the Mandarin fish (*Siniperca chuatsi*), walleye (*Stizostedion vitreum* = *Sander vitreus* (Mitchill, 1818)), gilthead seabream (*Sparus aurata* L., 1758), Japanese amberjack (*Seriola quinqueradiata*), spotted green pufferfish (*Tetraodon nigroviridis* Marion de Procé, 1822), house mouse (*Mus musculus* L., 1758), Nile tilapia (*Oreochromis niloticus* (L., 1758)), and human (*Homo sapiens* L., 1758).

lower expression levels in KT-1 males than in flutamide males. These results were quantitatively but not statistically supported by qPCR, although RNA quality and sample size may have both been issues in the qPCR analysis. We found no differences in behaviour-associated gene expression patterns, such as for prolactin, between 11-KT-implanted and flutamide-implanted males, despite the fact that males in these two treatment groups differed in the mean amount of nurturing behaviours that they performed during paternal care (Rodgers et al. 2013).

One of the primary differences that Rodgers et al. (2013) found between 11-KT-implanted and flutamide-implanted males was that flutamide-implanted males displayed more nurturing-type behaviours (such as egg fanning and the removal of dead eggs). Indeed, we found the same differences in the subset of individuals used here. Previously, it has been suggested that peptide hormones, specifically prolactin, play a significant role in regulating some of these behaviours. In the brown discus (*Symphysodon aequifasciata axelrodi* Schultz, 1960), males injected with prolactin increased the time that they spent fanning their eggs and increased mucus production, which males use to feed their fry (Blüm and Fiedler 1965). Similar mechanisms may also be at play in bluegill, as males implanted with bromocriptine, a dopamine receptor agonist that inhibits prolactin secretion, spent more time in prespawning behaviours, performed fewer nest defence behaviours, and spent significantly less time fanning eggs (Kindler et al. 1991). In our present study, we found that flutamide-implanted males exhibited more nurturing behaviours than 11-KT-implanted males, yet we observed no statistically significant differences in prolactin gene expression. Based upon these results, it is likely that the behavioural differences between males in the two treatments are the result of 11-KT inhibiting nurturing behaviours by mechanism(s) other than prolactin suppression. However, it would be interesting to examine how variation in prolactin influences nurturing behaviours of parental males without the confounding effects of the hormone implants used in this study.

The association between expression of immune-related genes and circulating androgen levels found in this study was not unexpected. Androgens, specifically T, have been implicated in shaping life-history trade-offs. While many studies have shown a decrease in immune response during the breeding season (Deerenberg et al. 1997; Suzuki et al. 1997; Casto et al. 2001; Pap et al. 2010), evidence regarding whether this response is primarily influenced by T is mixed (see Hasselquist et al. 1999; Saha et al. 2002; Greenman et al. 2005). One mechanism by which a relationship can occur is through androgens altering antigen presentation (Huber et al. 1999) or by binding to androgen receptors on T and B lymphocytes (Olsen and Kovacs 1996; Kanda et al. 1996). In fish, it has been shown that 11-KT can directly affect immune function by inhibiting lymphocyte proliferation (Cook 1994). Our data provide further support for the idea that androgens influence immune function, as all of the genes that were significantly suppressed by 11-KT are associated with immune system response. Because flutamide acts as an androgen receptor antagonist by competing with the native ligand for receptor binding sites, it is likely that the negative effects we observed on the expression of immune-related genes are occurring downstream from receptor binding. One such mechanism could be that flutamide is inhibiting T and 11-KT from binding to androgen receptors on lymphocytes, thus preventing suppression of immune system molecules like immunglobulins (Kanda et al. 1996).

The two transcripts that were most affected by 11-KT manipulation were direct matches for immunoglobulin M (IgM) heavy chain. Immunoglobulins are key components of the adaptive immune system and previous work has shown that testosterone suppresses both IgG and IgM in human peripheral blood monocular cells (Kanda et al. 1996). In teleosts, IgM is one of the dominant immunoglobulins, but how sex-specific steroids influence IgM gene expression is currently unclear. In the common carp (*Cyprinus carpio* L., 1758), high levels of T, but not 11-KT, were found to significantly decrease the number of IgM-secreting cells and the amount of IgM secreted in isolated cells from the spleen and head kidney (Saha et al. 2004). In rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)), individuals implanted with either T or 11-KT exhibited significant decreases in IgM levels in both blood plasma and external mucus, although the timing of this decrease differed between the two fluids, with effects observed over 1 to 7 days, respectively (Hou et al. 1999). In the present study, IgM gene expression was significantly affected by 11-KT only 3 days after hormone implantation. This result suggests that the suppressive effect of 11-KT on IgM gene expression in the brain occurs relatively quickly. However, how these changes translate into overall differences in immune function has not yet been fully addressed. Taken together, these studies show that the impact of 11-KT on IgM gene expression varies among species and that there are also tissue-specific differences regarding these effects. Given that IgM plays a significant role in the immune function of fish, future work should not only investigate the temporal effects of 11-KT but also how IgM gene expression is altered across tissues, such as the spleen and head kidney, which are both vital to immune function.

It is important to note that there are a few caveats for this study, one being the low sample size for our genetic analysis. This was primarily due to a combination of high nest abandonment rates and limited amounts of RNA that were of sufficient quality. Despite these issues, we were able to identify genes that differ in

expression levels between our treatment groups in the microarray analysis. However, future studies, with high-quality RNA and increased sample sizes, will likely detect additional genes associated with differences in parental care behaviours and hormone expression. Another caveat for this study was that the qPCR analysis did not verify our microarray results for the immunoglobulin gene. Immunoglobulin expression values from the qPCR data were significantly associated with the samples' RNA quality (i.e., RIN value) ($r = 0.83$, $p = 0.007$). In the array, we attempted to competitively hybridize individuals with similar RIN values to compensate for these differences and this methodology may explain at least some of the apparent discrepancy between the array and qPCR data sets. For the prolactin gene expression, there was no observed association between RNA quality and expression ($r = 0.52$, $p = 0.18$). Thus, it is possible that the relationship between immunoglobulin gene expression and RNA quality is coincidental. However, a recent study addressing degradation rates of various transcripts reported that those associated with immune genes degrade at a faster rate compared with the mean rate of all genes (Romero et al. 2014). Therefore, we cannot rule out the possibility that our immunogloblulin qPCR values are significantly confounded with RNA quality.

Overall, we found that parental male bluegill implanted with 11-KT exhibited significantly lower levels of IgM gene expression in the brain compared with flutamide-implanted males, despite the low sample size of the study. This result provides some evidence that in this species increased androgen levels influence immune system processes by potentially shunting available energetic resources away from immune function to reproduction. Males implanted with 11-KT also exhibited fewer nurturing behaviours than did flutamide-implanted males; however, these behavioural differences were not associated with prolactin gene expression. Taken together, our data suggest that parental male bluegill experience a significant trade-off between elevated 11-KT levels and both suppressed immune function and decreased levels of nurturing parental care behaviours.

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