Molecular mechanisms and the conflict between courtship and aggression in three-spined sticklebacks

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Abstract

In nature, animals often face conflicting demands. For example, breeding males must attract a mate but at the same time be ready to defend against rivals. The molecular mechanisms by which the brain resolves behavioural trade-offs are largely unknown. In this study, we compared the brain transcriptional responses of territorial male three-spined sticklebacks to a mating opportunity with a female and to a territorial challenge by a rival male. We focused on the diencephalon and the cerebellum, two regions of the brain implicated in courtship and aggression. There was a set of genes that were differentially expressed in response to both a courtship opportunity and a territorial challenge. Closer inspection of the direction of regulation revealed that genes that were downregulated in response to a courtship opportunity were upregulated in response to a territorial challenge and vice versa. Our study reveals some of the potential molecular mechanisms underlying behavioural trade-offs between sex and aggression, along with a possible solution to the conflict via social context-dependent gene regulation.

Keywords: behavioural syndrome, *Gasterosteus aculeatus*, gene expression, limited plasticity, microarray, sociogenomics, territoriality

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Introduction

Many animals in nature are constantly confronted with social stimuli. The ability to recognize and accurately respond to social stimuli is critical for survival and reproductive success. However, demands are not always clearly separated in time and space, and when they overlap, there can be conflicts between them. One of the most common and well-studied behavioural trade-offs is between courtship behaviour and territorial aggression (e.g. Kodric-Brown & Brown 1984; Candolin 1997; Santangelo *et al.* 2002; Dzieweczynski *et al.* 2009). For privileged access to resources, individuals vigorously defend a territory against intruders. However, aggression during territory defence can be detrimental if it is misdirected against potential mates. While the fitness costs and benefits of courtship and territory defence have been well studied, our understanding of

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the molecular mechanisms by which the brain might generate and/or resolve such conflicts is in its infancy (O'Connell & Hofmann 2011a).

A proximate mechanism that could generate trade-offs is when different behaviours are influenced by the same neural circuits or by same genes operating within neural circuits (Sih *et al.* 2004a). There is growing evidence that there are shared neural substrates underlying responses to social stimuli in vertebrates, that is the 'social behaviour network' (Newman 1999; Goodson 2005; O'Connell & Hofmann 2011b). Indeed, in mouse, neurons that are activated with aggression are colocalized with neurons associated with reproductive behaviour (Lin *et al.* 2011; Anderson 2012). There is also evidence for conflict between social stimuli at the molecular level. For example, the same genes that influence courtship behaviour also influence aggressive behaviour in fruit flies (Certel *et al.* 2007, 2010; Lin *et al.* 2011), potentially causing aggressiveness to 'spillover' to influence mating behaviour and vice versa (Sih *et al.* 2004a, b).

Early ethologists observing the behaviour of male three-spined stickleback fish (*Gasterosteus aculeatus*) noted that activities on the breeding grounds are highly dynamic: while nesting male three-spined sticklebacks in the field court females with conspicuous courtship displays, they are also constantly confronted by challenges such as sneakers and egg thieves and are primed to be very aggressive (Tinbergen 1951). These activities are important to reproductive success, but are potentially contradictory and are not always temporally or spatially separated from each other. Early theory motivated by studies of courtship and aggression in three-spined sticklebacks suggested that there are multiple 'motivations' or 'drives' that can come into conflict with one another and that sex and aggression are mutually inhibitory within individual males (Sevenster 1961; van den Assem 1967; Wilz 1972; Rowland 2000). For example, high aggression (aggression drive) might compromise a male's courting ability (sex drive) and might also come into conflict with parental care (parental drive; Sargent 1985). These observations prompt the hypothesis that the same molecular mechanisms are involved in responding to a courtship opportunity and a territorial challenge, and that there are mechanisms for resolving such complex and potentially contradictory behaviours.

In this study, we compared the brain gene expression profiles associated with territorial defence and courtship in male three-spined sticklebacks. Males were presented with either a territorial challenge by a rival male three-spined stickleback [data from (Sanogo *et al.* 2012)] or with a courtship opportunity with a gravid female three-spined stickleback. Brain gene expression was measured relative to an appropriate control group using microarrays. We focus on gene expression in diencephalon, which contains nodes with the social behaviour network (Ferris *et al.* 2008), for example hypothalamus and in the cerebellum, which has been implicated with sexual behaviour in other studies (Paredes-Ramos *et al.* 2011). We use these data to test the hypothesis that the neurogenomic responses to a courtship opportunity and a territorial challenge share common genes and biological processes. Our study suggests that males' response to conflicting demands is modulated by differential regulation of the expression of the same genes.

Materials and methods

The three-spined sticklebacks used in this study were wild-caught adults (one year of age) collected from a freshwater population in the Navarro River, California. The fish were maintained in the laboratory in 104-L tanks for at least 3 months prior to the experiments.

None of them had breeding experience in the laboratory. The water was filtered through particulate, UV, biological and charcoal filters. The adult fish were fed *ad libitum* with a mixture of bloodworms, brine shrimp and mysis shrimp. For more description of the laboratory conditions, see Sanogo *et al.* (2011).

Males were housed singly in tanks with a gravel bottom, a plant for refuge and a plastic nest box filled with sand and were provided with algae for nesting material. To induce breeding conditions, the photoperiod was set to 16:8 h light/dark and the temperature to 18 °C. All experiments were carried out in summer. Only males engaging in territorial and courtship behaviour (actively nest building) and showing nuptial red coloration on the throat were used in the experiments. On the morning of the experiment, opaque dividers were inserted between males' tanks to prevent visual interactions among neighbours.

Courtship opportunity experiment

Focal males with completed nests (*n* = 6) were presented with a live gravid female confined to a flask for 5 min (control, *n* = 6: empty flask). All of the males exhibited courtship behaviour towards females. Focal males were sacrificed 30 min after the female was introduced. By confining the female, we could prevent the pair from mating, thereby allowing us to capture genes associated with courtship rather than mating.

Territorial challenge experiment

Focal males with completed nests (*n* = 5) were presented with a smaller (~10%) free-swimming male intruder for 15 min (control, *n* = 5: no male). All of the males attacked the intruder. Focal males were sacrificed 30 min after the male was introduced. A detailed analysis of this experiment is reported in Sanogo *et al.* (2012). Here, we compare a subset of those results with the transcriptomic response to a courtship opportunity.

Brain dissection and RNA isolation

Fish were netted and quickly sacrificed by decapitation within seconds. Brains were immediately dissected, and we focus expression in the cerebellum and diencephalon. Structures codissected with the diencephalon included the thalamus, the hypothalamus, the posterior portion of the pituitary and the pineal gland, all of which have been implicated with aggression (Ferris *et al.* 2008). The teleost cerebellum is essential in discrete motor responses; however, recent studies have shown that this region of the brain is involved in other functions such as sexual and feeding behaviours

(Paredes-Ramos *et al.* 2011). The diencephalon was dissected by cutting the two lobes away from the cerebellum and removing the entire structure from the skull. The cerebellum was removed by cutting off the structure from the brain stem. RNA was extracted as described in Sanogo *et al.* (2011).

Microarray

The microarray chip used in both experiments is described in Sanogo *et al.* (2012).

Samples were labelled using the AGILENT QUICK AMP LABELING kits following the manufacturer's instructions. Detailed description of microarray hybridization in the territorial challenge experiment can be found in Sanogo *et al.* (2012). Briefly, in both experiments, up to 1 µg of total RNA from each sample was reverse transcribed into complementary RNA (cRNA), during which it was fluorescently labelled by incorporation of cyanine (Cy)-3-CTP (Cy3) or cyanine (Cy)-5-CTP (Cy5). Samples representing individual brain parts were labelled using either Cy3 or Cy5 dyes and competitively hybridized to the arrays. For hybridization, 200 ng of samples from control and experimental groups labelled with different dyes was mixed, fragmented and hybridized onto an Agilent 4 × 44K oligonucleotide microarray following the manufacturer's recommendations. Hybridization was performed within brain regions and between control and experimental replicates within each experiment. We used a 'balanced' design and controlled for dye effects by performing dye swaps on biological replicates (individuals). The microarray slides were scanned on an AXON 4000B scanner (Molecular Devices Corporation, Sunnyvale, CA, USA), and expression feature was extracted using GENEPIX PRO 6.0 software (Molecular Devices).

Data analysis

Within each experiment, we used R Bioconductor (R Development Core Team 2009) to identify differentially expressed genes between control and experimental groups. Brain regions were analysed separately. For example, to identify differentially expressed genes in cerebellum in response to a courtship opportunity, we compared gene expression in the cerebellum of males

that had been presented with a gravid female (experimental) vs. males that had not been presented with a female (control). Expression data from both experiments were analysed using the LIMMA single channel analysis as described in Smyth (2004). The territorial challenge experiment was analysed using rank product analysis in Sanogo *et al.* (2012). Importantly, the top genes of interest discussed in Sanogo *et al.* (2012; e.g. CGA, TSHB, POMC, PRL, CRHB) were also differentially expressed according to the LIMMA analysis. As preprocessing methods of gene expression intensities, we used 'loess' for within array normalization, 'aquantile' for between array normalization and 'normexpr' methods for background correction. A linear model was fit to the data that included treatment as a fixed factor. False discovery rate (FDR) was applied to control for multiple testing using the method of Benjamini & Hochberg (1995). To generate a longer gene list for bioinformatic analyses, we considered genes that were significant at the $P < 0.01$ level. The goal of this study was to compare the transcriptomic response between two social stimuli. The rationale for generating a longer gene list within each experiment was to improve our chances of evaluating similarities and differences between them. Although there are likely to be false positives within each list, the cross-experiment comparison should act as a filter to remove false positives. The probability that a false positive would appear in the overlap between both lists, for example, is very small. The differentially expressed genes were annotated using BioMart at ensembl.org. We used the package *Neatmap* (Rajaram & Oono 2010) to generate a heat map. We used hypergeometric tests within each brain region to assess whether commonalities between experiments was greater than expected due to chance (background: 22 518 genes).

The microarray data met the minimum information for microarray experiment (MIAME) criteria. The data from both experiments are available in GEO (Territorial challenge: Accession no. GSE32961; Courtship opportunity: Accession no. GSE74051.

Gene functional and gene ontology analyses

We conducted Gene Ontology (GO) enrichment analysis of each list of differentially expressed genes ($P < 0.01$) in

Table 1 Numbers of differentially expressed genes in diencephalon and cerebellum in response to a courtship opportunity and a territorial challenge at the FDR = 0.05 and $P < 0.01$ levels

| | FDR < 0.05 | | $P < 0.01$ | |
	Courtship opportunity	Territorial challenge	Courtship opportunity	Territorial challenge
Diencephalon	0	320	122	421
Cerebellum	216	0	1684	420

R Bioconductor using the package *GOstat* (Beissbarth & Speed 2004) as described in Sanogo *et al.* (2012). Stickleback human homologs were converted into ENTREZIDs for use in the package *GOstat*. The overrepresented biological processes (BPs), molecular functions (MFs) and cellular components (CCs) were determined using the function *hyperGtest* in the package *GOstat* with conditional testing which removes the effect of child GO terms before testing parents. We also performed GO on the list of genes that were differentially expressed in both experiments (both brain areas combined).

Results

Genomic response to a courtship opportunity

There were 122 DE transcripts (52 upregulated and 70 downregulated) in the diencephalon (Table S1) and 1684 transcripts (737 upregulated and 476 downregulated) DE in the cerebellum (Table S2) in response to a courtship opportunity (Table 1). The average log-fold difference was 0.58 in diencephalon and 0.48 in cerebellum. Among the most upregulated genes in the diencephalon was corticotropin releasing hormone binding protein (CRHBP). Among the most downregulated genes in the diencephalon were itchy E3 ubiquitin protein ligase (ITCH) and arginine vasopressin-induced 1 (AVPI1). The most upregulated genes in the cerebellum included mitochondrial ribosomal protein S10 (MRPS10) and leucine-rich repeat LGI family, member 3 (LGI). The most downregulated genes in the included ITCH and GABA A receptor, Beta 3 (GABRB3). These results suggest that there are different genomic responses to a social opportunity in the diencephalon and the cerebellum, but also some commonalities, for example ITCH.

Biological processes enriched in the diencephalon included GO terms related to behaviour such as maternal aggressive behaviour, and terms related to steroids such as steroid metabolic process, hormone metabolic process, oestrogen catabolic process, androgen metabolic process, luteinization and other processes related brain function such as synaptic transmission, dopaminergic and pyramidal neuron development (Table S3). In the cerebellum, the most enriched processes included adult behaviour, behaviour, male gonad development, male sex differentiation, ovarian follicle development, development of primary sexual characteristics and terms related to neuron development such as generation of neurons, neuron development and pyramidal neuron migration (Table S4).

Genomic response to a territorial challenge

In the diencephalon, there were 421 DE transcripts (152 upregulated and 269 downregulated, Table S5),

and in the cerebellum, there were 420 DE transcripts (239 upregulated and 181 downregulated, Table S6) in response to a territorial challenge. The average fold difference was 0.76 in diencephalon and 0.55 in cerebellum. There were more downregulated than upregulated genes in diencephalon, while the opposite was observed in the cerebellum. Among the differentially expressed genes in diencephalon were vitelline membrane outer layer 1 homolog (VMO1, down), glycoprotein hormones, alpha polypeptide (CGA, down) proopiomelanocortin (POMC, down), thyroid stimulating hormone (TSHB, down), ribosomal protein 4 (RPL4, up) and prolactin (PRL, down). The downregulation of PRL in response to a territorial challenge is particularly intriguing because another study found that administration of prolactin decreased courtship behaviour in three-spined sticklebacks (Páll *et al.* 2004). Other genes of interest include neurensin 1 (NRSN1), which was identified in a study of aggression in song sparrows (Mukai *et al.* 2009) and mitogen-activated protein kinase kinase 7 (MAP3K7), retinol binding protein 4 (RBP4) and leptin receptor overlapping transcript (LEPROT), which were differentially expressed in a mouse model of post-traumatic stress disorder (Muhie *et al.* 2015).

GO analysis points to some of the biological processes in the diencephalon and cerebellum in response to a territorial challenge. Some of the enriched biological processes in the diencephalon included terms related to response to steroid hormone, peptide hormone processing, hormone metabolic process, regulation of glucocorticoid secretion, dopamine uptake, noradrenergic neuron development and male sex differentiation (Table S7). In the cerebellum, the list of enriched biological processes included terms related to behaviour, development of primary sexual characteristics, male gonad development, ovarian follicle development, histone H3-K4 trimethylation, neuron projection morphogenesis and learning and memory (Table S8).

Comparing genomic responses to a courtship opportunity and a territorial challenge

The Venn diagram (Fig. 1) shows that there are commonalities between the neurogenomic response to a courtship opportunity and a territorial challenge at the molecular level. Indeed, hypergeometric tests within each brain region on the number of genes shared between the courtship opportunity and territorial challenge experiments revealed that the overlap in diencephalon was greater than expected by chance (diencephalon $P < 4.863e-04$, cerebellum $P < 0.18$, Fig. 1). Genes that were common to both experiments are in Table 2.

Closer inspection of the direction of regulation suggests that the same differentially expressed genes are

regulated differently in response to a courtship opportunity and a territorial challenge (Fig. 2). Some of the genes that were upregulated in response to a territorial challenge were downregulated in response to a courtship opportunity and vice versa. Several of the genes in this set have intriguing functions, such as ITCH, which was linked to anxiety-like behaviours in a QTL study (Kim *et al.* 2009); WDR48, which was linked to glutamatergic signalling in *C. elegans* (Dahlberg & Juo 2014); vasoactive intestinal peptide VIP, which has also been linked to courtship and aggression in birds (Goodson & Adkins-Regan 1999; Goodson *et al.* 1999; Kabelik *et al.* 2009; Goodson & Kingsbury 2013); neuroplastin NPTN, which has been linked to intelligence (Desrivieres *et al.* 2015); and schizophrenia (Saito *et al.* 2007) in humans; and PARK2, which is one of the top candidate genes for Parkinson's disease (Veeriah *et al.* 2010).

Discussion

Some of the same genes in the brain responded to a courtship opportunity and a territorial challenge (pleiotropy, Fig. 1). Interestingly, however, genes that were upregulated in response to a territorial challenge were downregulated in response to a courtship opportunity
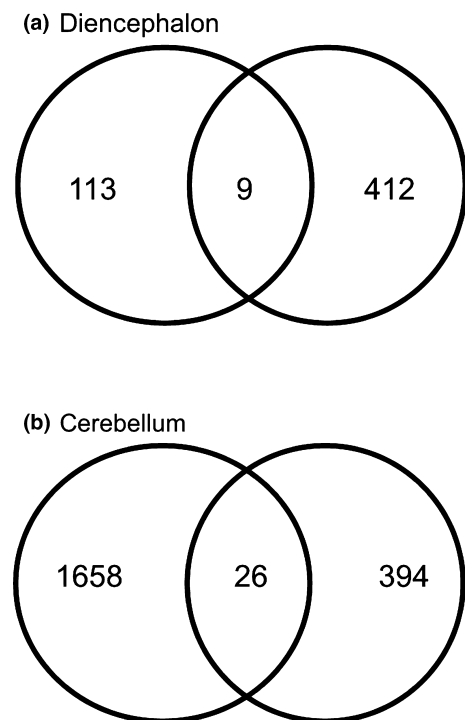
(a) Diencephalon

(b) Cerebellum

Fig. 1 Venn diagrams showing the overlap between the lists of differentially expressed genes ($P < 0.01$) in response to a courtship opportunity (left circle) and a territorial challenge (right circle) in (a) diencephalon and (b) cerebellum.

and vice versa (Fig. 2). These results suggest that the conflict between the molecular response to a courtship opportunity and a territorial challenge is at least partially resolved via gene regulation. They suggest that when males engage in territorial defence, expression of genes related to courtship behaviour decreases (and vice versa). Genes involved in managing trade-offs at the molecular level are likely to include transcription factors acting within gene regulatory networks to up- or downregulate expression, depending on the animal's response.

Areas within the diencephalon were socially responsive, especially to a territorial challenge. This finding is not altogether surprising given that the diencephalon includes the hypothalamus, which includes key nodes within the social behaviour network (O'Connell & Hofmann 2011b). Slightly more surprising is that in response to a courtship opportunity, more differentially expressed genes were detected in cerebellum [a brain region typically associated with fine motor control and movement (Morton & Bastian 2004)] than in diencephalon. However, areas within the cerebellum have been linked with sexual behaviour in other species, for example rats (Manzo *et al.* 2008), cichlids (Burmeister *et al.* 2005) and quail (Cornil *et al.* 2006), and the cerebellum might be involved in the fine movement zigzag dance that males display towards females during courtship.

There was a suite of genes that were regulated in opposite directions in response to a territorial challenge and a courtship opportunity (Table 2). Among those genes is VIP, which was downregulated in the cerebellum in response to a territorial challenge and upregulated in the cerebellum in response to a courtship opportunity. VIP is a particularly interesting candidate because it has been associated with the modulation of aggression in other species (e.g. Goodson & Adkins-Regan 1999; Goodson *et al.* 2012). For example, VIP-producing neurons in the anterior hypothalamus promote aggression (nest defence) in birds in a social context-dependent manner (Goodson *et al.* 2012). The finding that a set of genes is regulated in opposing directions in response to different social stimuli is not unprecedented. For example, Cummings *et al.* (2008) identified a set of genes that was 'upregulated' while female swordtails interacted with attractive males and 'downregulated' when interacting with other females and vice versa. Similarly, work on *Drosophila* has shown that males' responses to other males and other females often involve the same molecular mechanisms (Certel *et al.* 2007, 2010; Lin *et al.* 2011).

If there are shared molecular mechanisms associated with opposing behavioural responses to different social stimuli, there must be a way to negotiate between them;

Table 2 Genes that were differentially expressed in both experiments separated by brain region

Transcript ID	Territorial challenge	Courtship opportunity	Annotation	Description
(A) Diencephalon				
ENSGACT00000000115	Up	Up	F3 (1 of 3)	Coagulation factor III (thromboplastin, tissue factor)
ENSGACT00000000118	Up	Up	F3 (2 of 2)	Coagulation factor III (thromboplastin, tissue factor)
ENSGACT00000000119	Up	Up	F3 (3 of 3)	Coagulation factor III (thromboplastin, tissue factor)
ENSGACT00000001576	Up	Down	PURB	Purine-rich element binding protein B
ENSGACT00000007796	Up	Down	ITCH	Itchy E3 ubiquitin protein ligase
ENSGACT00000010268	Up	Down	GSKIP	GSK3B interacting protein
ENSGACT00000022081	Up	Down	Novel transcript	
ENSGACT00000016398	Down	Up	PARK2	Parkinson protein 2, E3 ubiquitin protein ligase (Parkin)
ENSGACT00000024271	Down	Up	NR5A1	Nuclear receptor subfamily 5, group A, member 1
(B) Cerebellum				
ENSGACT00000008470	Up	Up	NPTN	Neuroplastin
ENSGACT00000003310	Up	Up	MPLKIP	M-phase specific PLK1 interacting protein
ENSGACT00000011405	Up	Up	ITFG3	Integrin alpha FG-GAP repeat containing 3
ENSGACT00000013369	Down	Up	CHD4	Chromodomain helicase DNA binding protein 4
ENSGACT00000005701	Down	Up	Novel transcript	
ENSGACT00000027746	Down	Up	CYTB	Cytochrome b (mitochondrion)
ENSGACT00000001688	Down	Up	VIP	Vasoactive intestinal peptide
ENSGACT00000007347	Down	Up	SEP5	Septin 5
ENSGACT00000025253	Down	Up	SYNGR2	Synaptogyrin 2
ENSGACT00000016749	Down	Up	WDR48	WD repeat domain 48
ENSGACT00000000221	Down	Up	CAMLG	Calcium modulating ligand
ENSGACT00000020115	Down	Up	WDR45B	WD repeat domain 45B
ENSGACT00000007631	Down	Up	PTP4A2	Protein tyrosine phosphatase type IVA, member 2
ENSGACT00000009534	Down	Down	TAX1BP1	Tax1 (human T-cell leukaemia virus type I) binding protein 1
ENSGACT00000001516	Down	Down	SLC25A34	Solute carrier family 25, member 34
ENSGACT00000013274	Down	Down	POC5	POC5 centriolar protein homolog (Chlamydomonas)
ENSGACT00000011783	Down	Down	ARMC2	Armadillo repeat containing 2
ENSGACT00000001976	Down	Down	MYSM1	Myb-like, SWIRM and MPN domains 1
ENSGACT00000008785	Down	Down	CDK19	Cyclin-dependent kinase 19
ENSGACT00000001930	Down	Down	TAF1B	TATA box binding protein (TBP)-associated factor, RNA polymerase I, B, 63 kDa
ENSGACT00000010815	Down	Down	PDE1A	Phosphodiesterase 1A, calmodulin-dependent
ENSGACT00000015480	Down	Down	SETDB1	SET domain, bifurcated 1
ENSGACT00000012593	Down	Down	CSAD (1 of 1)	Cysteine sulfinic acid decarboxylase
ENSGACT00000012633	Down	Down	CSAD (2 of 2)	Cysteine sulfinic acid decarboxylase
ENSGACT00000006262	Down	Down	EIF5	Eukaryotic translation initiation factor 5
ENSGACT00000012162	Down	Down	PTPN3	Protein tyrosine phosphatase, non-receptor type 3

otherwise, territories would go undefended and no one would mate. Our findings suggest that these conflicts are resolved at a higher, systems level, by transcriptional gene regulation. Indeed, our findings are consistent with studies of honeybees showing social context-specific effects of transcription factors (Chandrasekaran *et al.* 2011). Lags in gene regulation could potentially explain why an individual's behaviour can 'spillover' to influence behaviour in other contexts – maybe the reason why males attack rather than court an attractive female, for example, is because he recently fought with a male, and gene regulation does not occur fast enough to put a break on his aggression.

The overlapping gene sets in response to the two social stimuli are all the more remarkable considering that there were subtle differences between the two experiments. For example, in the territorial challenge experiment, the male conspecific was free swimming, while in the courtship opportunity experiment, the female was confined to a flask. Therefore, there was an opportunity for males to be exposed to both olfactory and visual cues in the territorial challenge experiment, but only to visual cues in the courtship opportunity experiment. Similarly, although in both experiments, males were sacrificed 30 min after the conspecific was introduced, in the territorial challenge experiment, the conspecific was present for 15 min, while in the courtship opportunity experiment, the conspecific was present for 5 min. Females were presented for 5 min because previous studies have shown that males
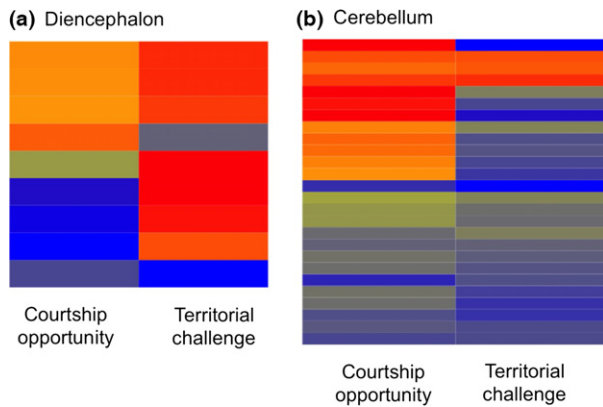
(a) Diencephalon   **(b)** Cerebellum

Fig. 2 Heatmaps comparing the regulation of the genes that were differentially expressed in response to both a courtship opportunity and a territorial challenge in (a) Diencephalon and (b) Cerebellum. The column on the left indicates how the shared genes were expressed in response to a courtship opportunity; the column on the right indicates how the shared genes were expressed in response to a territorial challenge. Note that many of genes that were upregulated in response to a courtship opportunity were downregulated in response to a territorial challenge and vice versa. Red, upregulated; blue, downregulated; yellow, NS.

quickly habituate to females, but take longer to habituate to males (Bell & Peeke 2012). We speculate that the duration of exposure to the stimulus might have affected the stimulus' salience – the territorial challenge might have been more salient than the courtship opportunity because the territorial challenge was present for longer. It will be useful for future studies to examine the effects of duration of exposure to a social stimulus on gene expression. However, for the purposes of this study, which was to look for commonalities between the transcriptomic response to two different social stimuli, the fact that there were subtle differences between the two experiments implies that the comparison between experiments is conservative.

Studies on the mechanisms underlying the sex-aggression conflict have tended to focus on whether a male should court or attack a female in the context of mate recognition, rather than comparing males' responses to a threat vs. an opportunity (Certel *et al.* 2007; Andrews *et al.* 2014). Similarly, there is a behavioural literature on the sex-aggression trade-off that has tended to focus on the conflict in real time. That is, when males are presented with a male and female simultaneously, a common result is that the presence of competitors causes males to decrease courtship (Kodric-Brown & Brown 1984; Candolin 1997; Santangelo *et al.* 2002; Dzieweczynski *et al.* 2009). A promising direction for future studies is to compare the neurogenomic response to a threat and an opportunity presented simultaneously vs. separately. Careful experiments could potentially tease apart genes related to

courtship, genes related to aggression and genes related to managing the conflict between them.

From a behavioural point of view, responding to a territorial intruder vs. a potential mate involves very different responses – male three-spined sticklebacks attack a territorial intruder, vigorously defending their territory. Males actively court and encourage gravid females to visit their nest, and if a male is already engaged in courtship with one female, the approach of a second female could represent a social challenge. That being said, there are often elements of aggression involved in responding to a gravid female for a variety of reasons, for example if she is not receptive to his courtship attempts, if she is not attractive or if she is perceived as a nest predator rather than a potential mate. Indeed, based on observations of three-spined stickleback behaviour, Tinbergen initially envisioned a close mechanistic link between sex and aggression (Tinbergen 1951), a link supported by their overlapping neural circuits in mice (Anderson 2012). This experiment offers insights into the molecular mechanisms underlying behavioural trade-offs and a possible solution to the conflict via social context-dependent gene regulation.

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Y.O.S. executed the experiments, analysed the data and wrote the study; A.M.B. designed the experiments, analysed the data and wrote the study.

Data accessibility

The data from the territorial challenge experiment are available in GEO (Accession no. GSE32961). The data from the courtship opportunity experiment have been deposited in GEO (Accession no. GSE74051, to be released in October 2016).

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 List of DE genes in diencephalon in response to a courtship opportunity at raw p < 0.01.

Table S2 List of DE genes in cerebellum in response to a courtship opportunity at raw p < 0.01.

Table S3 GO processes enriched in diencephalon in response to a courtship opportunity.

Table S4 GO processes enriched in cerebellum in response to a courtship opportunity.

Table S5 List of DE genes in diencephalon in response to a territorial challenge at raw p < 0.01.

Table S6 List of DE genes in cerebellum in response to a territorial challenge at raw p < 0.01.

Table S7 GO processes enriched in diencephalon in response to a territorial challenge.

Table S8 GO processes enriched in cerebellum in response to a territorial challenge.