Brain Transcriptional Profiles of Male Alternative Reproductive Tactics in Bluegill Sunfish

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Abstract

Bluegill sunfish are one of the classic systems for studying male alternative reproductive tactics (ARTs) in teleost fishes. In this species, there are two distinct life histories: parental and cuckolder, encompassing three reproductive tactics, parental, satellite, and sneaker. The parental tactic is fixed, whereas individuals who enter the cuckolder life history transition from the sneaker to the satellite tactic as they grow. For this study, we used RNAseq to characterize the brain transcriptome of the three male tactics during spawning to identify gene categories associated with each tactic and identify potential candidate genes influencing their different spawning behaviors. We found that sneaker males had higher levels of gene differentiation compared to the other two tactics, suggesting that life history does not exclusively drive differential gene expression. Sneaker males had high expression in ionotropic glutamate receptor genes, specifically AMPA receptors, which may be important for increased working spatial memory while attempting to cuckold nests in bluegill colonies. We also found significant expression differences in several candidate genes involved in ARTs that were previously identified in other species and suggest a previously undescribed role for cytosolic 5'-nucleotidase II (*nt5c2*) in influencing parental male behavior during spawning.

Introduction

Understanding the genetic mechanisms influencing variation in behavior can provide insight into how different behavioral phenotypes within populations evolve and are maintained. One important area of research on behavioral phenotypes focuses on alternative reproductive tactics (ARTs), which are found in a wide array of taxa (Gross 1996; Mank & Avise 2006; Taborsky *et al.* 2008; Taborsky & Brockman 2010). ARTs typically consist of larger males practicing a "territorial" tactic that maintain and protect breeding territories and smaller "sneaking" males that sneak fertilizations rather than compete with territorial males (Taborsky 1998). The mechanisms underlying the expression of ARTs can differ significantly across species. In some cases, tactics are fixed for life (fixed tactics) (Taborsky 1998) and often represent distinct life histories. Fixed tactics can occur through either inherited genetic polymorphisms (Lank *et al.* 1995; Shuster & Sassaman 1997), condition-dependent switches that are triggered prior to sexual maturation (Taborsky 1996; Gross 1996; Gross & Repka 1998), or a combination of genetic and environmental factors (Piché *et al.* 2008, Neff & Svensson 2013). In other cases, individuals can exhibit different tactics throughout their reproductive life, either as they grow or in response to changing social context (plastic tactics or status-dependent tactics) (Gross 1996; Taborsky 1996; Taborsky *et al*. 2008). Recent advances in genome sequencing, such as RNA sequencing (RNAseq), now allow behavioral ecologists to explore what genetic pathways contribute to behavioral variation among mating tactics and examine if the pathways differ among species or between individuals that exhibit fixed versus plastic tactics.

57 Over the past few years there have been numerous studies examining how
58 differences in gene expression correlate with behaviors adopted by male ARTs
59 (Aubin-Horth *et al*. 2005; Renn *et al.* 2008; Fraser *et al.* 2014; Schunter *et al.* 2014;
60 Stiver *et al.* 2015). Most of these studies have found a large number of genes within
61 the brain that vary in expression among tactics during mating. For example, a recent
62 study examining gene expression differences in the ocellated wrasse (*Symphodus*
63 *ocellatus*) found 1,048 differentially expressed genes when comparing sneakers to
64 two other male tactics (nesting and satellite) and to females (Stiver *et al.* 2015). In
65 the black-faced blenny (*Tripterygion delaisi*), RNAseq identified approximately 600
66 transcripts differentially expressed within the brains of sneaker versus territorial
67 males (Schunter *et al.* 2014). In a third study, approximately 2,000 transcripts were
68 differentially expressed between intermediately-sized sailfin molly (*Poecilia*
69 *latipinna*) males that primarily perform courtship behaviors compared to small
70 males that only perform sneaking behaviors (Fraser *et al.* 2014). Changes in social
71 context also led to a larger response (i.e. changes in gene expression) in
72 intermediate-sized males that show higher levels of tactic plasticity when compared
73 to small sneaker males (Fraser *et al*. 2014), suggesting that genes driving neural
74 response during mating may differ between plastic and fixed tactics.
75 With the increase in genomic studies examining differential gene expression
76 among male ARTs, there are a growing number of candidate genes suggested to
77 drive the behavioral differences among tactics. Schunter *et al*. (2014) proposed a list
78 of potential candidate genes based on a number of studies that included
79 gonadotropin releasing hormone (*gnrh*), arginine vasotocin (*avt*), cytochrome P450

family 19 subfamily A polypeptide 1 (*cyp19a1*), ependymin (*epd*), galanin (*gal*), stomatostatin (*sstr1* and *sstr3*), and early growth response 1 (*egr1*). Many of these genes are involved in hormone regulation and mating behavior, and differences in expression levels have been observed among mating tactics in different fish species (Table 1). For example, the product of the *cyp19a1b* gene is aromatase B, a key enzyme responsible for the conversion of androgens to estrogens within radial glial cells of adult fish (Forlano and Bass 2005; Le Page *et al.* 2010). *Cyp19a1* plays an important role in sex determination and sex change in fish (Nakamura & Kobayashi 2005; Black *et al.* 2005; Marsh *et al.* 2006) and higher levels of gene expression have been observed in territorial males compared to sneaker males in peacock blennies (*Salaria pavo*) (Gonçalves *et al.* 2008), black-faced blennies (*Tripterygion delaisi*) (Schunter *et al*. 2015), and an African cichlid (*Astatotilapia burtoni*) (Renn *et al.* 2008). As more genomic data become available, the number of candidate genes in this list will likely increase and evaluating gene responses across taxa will aid in determining whether similar genetic pathways drive ART behaviors across different species.

One of the best-studied fish species with male ARTs is the bluegill sunfish (*Lepomis macrochirus*). In this species, males have two distinct life histories: parental and cuckolder. In Lake Opinicon (Ontario, Canada), parental males mature at around seven years old and construct nests, court females, and provide care to young (Gross 1982). Cuckolder males mature at a significantly younger age, around two years old (Gross 1982). Rather than competing with parental males for access to females, cuckolders initially use a “sneaking” tactic to dart in and out of nests

103 while parental males and females are spawning. As they grow, typically around an
104 age of 4 years, cuckolder males transition into "satellite" males by taking on female-
105 like coloration and behaviors (Dominey 1980; Gross 1982). Satellite males use this
106 female mimicry to deceive a parental male that he has two true females in his nest
107 (Neff & Gross 2001). The parental and cuckolder life histories are fixed – once a
108 male adopts the parental or cuckolder life history, he remains in that life history for
109 life (Gross & Charnov 1980). However, within the cuckolder life history, mating
110 tactics are ontologically plastic, with males apparently transitioning from the
111 sneaker tactic to the satellite tactic as they age (Gross & Charnov 1980).

112 While the spawning behavior, reproductive success, and hormone profiles of
113 bluegill have been studied extensively (Gross & Charnov 1980; Kindler *et al.* 1980;
114 Kindler *et al*. 1991; Neff 2001; Neff 2004; Knapp & Neff 2007), the genetic factors
115 influencing behavioral differences during spawning are less clear (Partridge *et al*.
116 2015). Thus, for this study, we used RNAseq to characterize the brain transcriptome
117 of the three spawning male tactics (parental, sneaker, and satellite), in addition to
118 non-spawning parental males, to examine how differences in gene expression may
119 relate to behavioral variation among the tactics. Specifically, we (1) assessed
120 whether or not there is a greater difference in gene expression profiles between
121 fixed tactics (parental versus the two cuckolder tactics) than between tactics within
122 a plastic life history (sneaker versus satellite), (2) identified specific gene categories
123 that are expressed for each tactic, and (3) examined the expression of potential
124 candidate genes associated with ARTs from other fish species to determine if they
125 also differentiate the ARTs in bluegill.

126

127 **Methods and Materials**

128 Bluegill Sampling

129 In June 2013, bluegill sunfish were collected from Lake Opinion near Queen's
130 University Biological Station (QUBS), Elgin, Ontario, Canada. A total of 12 parental
131 males, 12 sneaker males, 13 satellite males, and 12 females were collected in the act
132 of spawning directly from the bluegill colony. An additional 12 non-nesting parental
133 males were collected four days prior to spawning (as determined once spawning at
134 these colonies began). Individuals were euthanized using clove oil, total body length
135 was measured, and brains were immediately dissected out and stored in RNAlater
136 (Life Technologies, Carlsbad, CA). Brains remained in RNAlater at 4°C for 24 hours
137 and were then transferred to fresh cryovials, flash frozen, and kept in liquid
138 nitrogen until they were transported on dry ice to the University of Western
139 Ontario. Samples were then stored at -80°C until RNA extraction.

140

141 Total RNA Extraction

142 Total RNA was extracted using a standard Trizol (Life Technologies,
143 Carlsbad, CA) extraction. RNA was submitted to the London Genomics Center at the
144 University of Western Ontario and quality was assessed using a 2100 Bioanalyzer
145 (Agilent Technologies, Palo Alto, CA). Four individuals from each group (spawning
146 parental males, non-spawning parental males, sneaker males, satellite males, and
147 females), for a total of 20 individuals, were submitted to the Michigan State
148 University Research Technology Support Facility - Genomics Center for cDNA

149 Library Construction and Sequencing. Individuals used for this study had RIN (RNA
150 Integrity Number) values ranging from 9.2-9.9.
151
152 cDNA Library Construction and Sequencing
153 The cDNA libraries were constructed for each individual using Illumina
154 TrueSeq Stranded mRNA Library Preparation Kits LT (Illumina, San Diego, CA), with
155 each individual receiving a uniquely identifiable index tag. The quality of each
156 library was evaluated and the 20 individuals were multiplexed into a single sample
157 that was subsequently run on two lanes of an Illumina HiSeq2500 Rapid Run flow
158 cell (v1). Sequencing was performed on paired end 2 x 150 bp format reads and
159 bases were called using Illumina Real Time Analysis software (v1.17.21.3). Reads
160 from each individual were identified based on their unique index tag, separated, and
161 converted to fastq files using Illumina Bcl2fastq v1.8.4. Sequencing produced an
162 average of 14.5 million reads per individual, with over 90% of the reads having a Q-
163 score >30.
164
165 *De novo* Transcriptome Assembly and Reference Transcriptome
166 Prior to assembly, read quality was assessed using FastQC
167 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Nucleotides whose
168 quality score was below PHRED=2 were trimmed using Trimmomatic version 0.32
169 (Bolger *et al*. 2014), following recommendations from MacManes (2014). The
170 reference transcriptome was assembled *de novo* using Trinity version 2.04 (Haas *et*
171 *al.* 2013, Grabherr *et al*. 2011). One representative of each of the five groups

172 (spawning parental male, non-spawning parental male, sneaker male, satellite male,
173 and female) was used to construct a combined reference transcriptome. The five
174 representatives selected for the reference were the individuals with the highest
175 number of reads within their group and, a total of 85 million paired-end reads were
176 assembled. The assembly was conducted with both normalized and non-normalized
177 reads and normalization was performed using Trinity's *in silico* normalization
178 program. To test the completeness of the transcriptome, reads from samples not
179 used in the assembly were mapped back to the transcriptome using Burrows-
180 Wheeler Aligner (bwa)-mem version 0.7.12 (Li 2013), and >90% of those reads
181 aligned, which is comparable to the rate of mapping for the individuals used in the
182 assembly (92%).
183 TransDecoder was used to identify protein-coding regions within the
184 assembled transcriptome. Transcripts that contained protein-coding regions or
185 transcripts that blasted to compete coding sequences (cds) and non-coding RNA
186 (ncRNA) from *Tetraodon nigroviridis, Lepisosteus oculatus, Xiphophorus*
187 *maculatus, Oryzias latipes, Takifugu rubripes, Latimeria chalumnae, Astyanax*
188 *mexicanus, Danio rerio*, or *Poecilia formosa* (downloaded from Ensembl) comprised
189 the reference transcriptome used for both read alignment and to estimate transcript
190 counts.
191
192 Read Alignment and Transcript Counts
193 Reads from each individual were separately aligned to the reference
194 transcriptome using bwa-mem 0.7.10 (Li 2013). At least 85% of all reads from each

195 individual mapped back to the reference, with the majority aligning 90% of reads or
196 higher. The sequence alignment/map (sam) files were then converted to a binary
197 format (bam) using Samtools 0.1.19 (Li *et al.* 2009). Transcript counts for each
198 individual were obtained using the program eXpress 1.5.1 (Roberts & Pachter
199 2013). Differential gene expression was determined using the R statistical package
200 edgeR 3.6.8 (Robinson *et al.* 2010). Low abundance transcripts were filtered out,
201 leaving 19,804 transcripts for differential analysis. Transcript counts were
202 normalized to account for differences in cDNA library size among individuals and
203 dispersion parameters were estimated using Tagwise dispersion estimates.
204 Differences in gene expression comparing paired treatments were calculated using
205 an Exact-test for binomial distribution. Genes with p-values lower than 0.05 after
206 false discovery rate (FDR) correction were determined to be statistically significant.
207 Multidimensional clustering analysis was used to cluster individuals together based
208 on the biological coefficient of variation.
209
210 Gene Annotation and Enrichment Analysis
211 Both the reference transcriptome and transcripts differentially expressed
212 among groups were blasted using Blastx against a custom-assembled fish protein
213 database. This database consisted of Ensembl protein databases of 13 different fish
214 species: Amazon molly (*Poecilia formosa*), zebrafish (*Danio rerio*), blind cave tetra
215 (*Astyanax mexicanus*), cod (*Gadus morhua*), coelancanth (*Latimeria chalumnae*),
216 Japanese pufferfish (*Takifugu rubripes*), sea lamprey (*Petromyzon marinus*), medaka
217 (*Oryzias latipes*), platyfish (*Xiphophorus maculatus*), spotted gar (*Lepisosteus*

218 *oculatus*), three-spined stickleback (*Gasterosteus aculeatus*), green-spotted
219 pufferfish (*Tetradon nigroviridis*), and Nile tilapia (*Oreochromis niloticus*). Blast hits
220 with e-values less than $1x10^{-10}$ were considered significant. Ensembl IDs from the
221 blast hits were then converted into GO term identifiers using Biology Database
222 Network (bioDBnet) (http://biodbnet.abcc.ncifcrf.gov/db/dbFind.php).
223 For purposes of gene annotation and enrichment analysis, we focused on
224 transcripts within the reference transcriptome that were not filtered out of the data
225 set due to low transcript expression (total of 19,804 transcripts). To examine which
226 GO terms were significantly enriched within this set, unique Ensembl IDs from
227 Blastx were converted to Ensemble IDs associated with stickleback homologs using
228 the R package biomaRt 2.20.0. Enrichment analysis was then conducted on the
229 homologs using the BioMart portal (http://central.biomart.org/enrichment).
230 For the transcripts that were differentially expressed among behavioral
231 groups, enrichment analysis was conducted using a Fisher Exact test to examine
232 whether the proportion of genes within each GO category was significantly higher
233 than what would be expected based upon the proportion of genes assigned to that
234 GO term within the reference transcriptome. To ensure adequate statistical power,
235 only GO terms with at least 10 transcripts within each category were included in the
236 statistical analysis. A FDR correction was applied to control for multiple testing and
237 GO terms with p-values < 0.05 were considered to be significant.
238
239
240

241 **Results**

242 Reference Transcriptome

243 This study presents the first reference transcriptome for the brain of bluegill
244 sunfish. The fully assembled transcriptome consisted of 272,189 transcripts. Of
245 these, 72,189 transcripts contained complete coding sequences or blasted to cds or
246 ncRNA from the customized Ensembl fish database. These 72,189 transcripts were
247 then used as the reference transcriptome for alignment and mapping. The mean
248 transcript length within the reference transcriptome was 2,024 bp, with N50 =
249 3,106 bp and N90 = 1,018 bp. The largest transcript consisted of 27,880 bp.
250 Approximately 82% of the transcripts had only one isoform, while 18% (12,951
251 transcripts) had two or more isoforms.

252 For GO enrichment analysis, we only examined the 19,804 transcripts within
253 the reference transcriptome that passed the filtering process. Of these, 18,108 had
254 significant Blastx hits with Ensembl gene IDs (Table S1, Supporting Information), of
255 which 12,224 transcripts had stickleback homologs that could be used to examine
256 GO term enrichment for bluegill sunfish compared to the stickleback genome. The
257 GO terms with significant enrichment included translation, catabolism, vesicle-
258 mediated transport, biosynthesis, small molecule metabolism, and generation of
259 precursor metabolites and energy (Fig. 1 A & B).

260

261 Differential Gene Expression among Groups

262 Based on the biological coefficient of variation, sneaker males grouped separately
263 from the other male tactics. However, non-spawning parental males, spawning

264 parental males, and satellite males showed a large amount of overlap in how
265 transcripts varied in their expression levels (Fig. 2). The largest number of
266 differentially expressed genes was observed when comparing spawning parental
267 males to sneaker males, followed by satellite males compared to sneaker males,
268 spawning parental males compared to satellite males, and then spawning parental
269 males compared to non-spawning parental males (Table 2). Analysis of sex
270 differences in differential expression will be presented in a companion paper
271 (Partridge et al. *in preparation*).
272
273 *Differential Expression between Life Histories*
274 Spawning Parental Males versus Sneaker Males. A total of 9,279 transcripts were
275 differentially expressed between parental males and sneaker males. Of these, 4,537
276 showed higher expression in parental males (Table S2, Supporting Information),
277 and 4,742 transcripts showed higher expression in sneaker males (Table S3,
278 Supporting Information). Enrichment analysis of GO terms associated with
279 differentially expressed genes showed that the biological processes most enriched
280 in parental males included translational initiation, translation elongation, and
281 oxidation-reduction processes (Table S4, Supporting Information). The molecular
282 function GO terms associated with translational processes included structural
283 constituents of the ribosome and translation initiation factor activity (Table S4,
284 Supporting Information). Biological processes enriched with genes displaying
285 higher expression in sneaker males included ion transport, hemophilic cell adhesion
286 (primarily due to protocadherin- and cadherin-related genes), the ionotropic

287 glutamate receptor signaling pathway, protein phosphorylation, and synaptic
288 transmission (Fig. 3A). Similarly, significantly enriched molecular function terms
289 included ionotropic glutamate receptor activity and protein binding (Table S4,
290 Supporting Information).
291
292 Spawning Parental Males versus Satellite Males. A total of 1,141 transcripts were
293 differentially expressed between parental males and satellite males. Of these, 676
294 displayed higher expression in parental males (Table S5, Supporting Information)
295 and 465 showed higher expression in satellite males (Table S6, Supporting
296 Information). Only one GO term related to biological processes was enriched in
297 parental males compared to satellite males and this was oxidation-reduction
298 processes. Significantly enriched molecular functions included iron ion binding,
299 oxidoreductase activity, and heme binding. Biological process terms enriched with
300 genes showing higher expression in satellite males included ion transport, and
301 enriched molecular function terms included nucleic acid binding, ion channel
302 activity, and GTP binding (Table S4, Supporting Information).
303
304 *Differential Expression within Life Histories*
305 Satellite Males verses Sneaker Males. There were 2,590 transcripts differentially
306 expressed between satellite males and sneaker males. Of these transcripts, 1,261
307 showed higher expression in satellite males (Table S7, Supporting Information), and
308 1,329 showed higher expression in sneaker males (Table S8, Supporting
309 Information). Biological processes enriched with genes displaying higher expression

310 in satellite males included translation, embryo development, and cell cycle
311 regulation. Molecular processes enriched in satellite males included structural
312 constituents of the ribosome, heme binding, and oxidoreductase activity (Table S4,
313 Supporting Information). Transcripts showing higher expression in sneaker males
314 were involved in biological processes related to ionotropic glutamate receptor
315 signaling pathways and mRNA processing (Fig. 3B). Molecular functions were
316 primarily related to ionotropic glutamate receptor activity and protein binding
317 (Table S4, Supporting Information).
318
319 Spawning Parental Males verses Non-Spawning Parental Males. A total of 137
320 transcripts were differentially expressed between spawning and non-spawning
321 parental males. The majority of these transcripts (132 transcripts) showed higher
322 expression in spawning males (Table S9, Supporting Information). Genes with the
323 highest expression in spawning males compared to non-spawning males were MHC
324 II antigen beta chain, cytosolic 5'-nucleotidase II (*nt5c2*), cAMP responsive element
325 modulator (*crem*), cysteine dioxygenase type 1 (*cdo1*), and an uncharacterized
326 protein. Only eight transcripts showed higher expression in non-spawning parental
327 males. These were nuclear receptor subfamily 1 group D member 4b (*nr1d4b*),
328 neuronal tyrosine-phosphoinositide-3-kinase adaptor 2 (*nyap2*), sphingosine-1-
329 phosphate receptor 4 (*s1pr4*), gamma-aminobutyric acid A receptor beta 3 (*gabrb3*),
330 and four uncharacterized proteins. Due to the limited number of transcripts
331 differentially expressed between these two groups, the number of transcripts

332 assigned to each GO term was too small to have adequate statistical power to
333 perform an enrichment analysis for this comparison.

334 *Potential Candidate Genes Associated with ART Spawning Behavior*

335 We observed differential expression in a number of transcripts previously
336 identified as potential candidate genes (described in Table 1) associated with
337 differences in ART spawning behaviors (Table 3). In our data set, the candidate
338 genes cytochrome P450 family 19 subfamily A polypeptide 1b (*cyp19a1b*),
339 ependymin (*epd*), and galanin (*gal*) showed higher expression in parental males
340 compared to sneaker males. *Epd* also had higher expression in satellite males
341 compared to sneakers. Early growth response 1 (*egr1*) showed higher expression in
342 both satellite and sneaker males relative to spawning parental males. Somatostatin
343 1 (*sstr1*) showed higher expression in sneaker males compared to satellite males,
344 but no differences in other comparisons between tactics. No differences in
345 expression related to gonadotropin releasing hormone (*gnrh*), arginine vasotocin
346 (*avt*), or somatostatin 3 (*sstr3*) were observed between any of our groups.

347 In addition to these previously identified candidate genes, transcripts that
348 displayed some of the highest differences in expression between parental males and
349 all other male phenotypes (including non-spawning males) were related to cytosolic
350 5'-nucleotidase II (*nt5c2*). Multiple isoforms were expressed, with log2 fold changes
351 ranging from 1.5 – 4.8 times higher in parental males compared to other male
352 groups (Fig. 4). Consistent with the finding for GO term enrichment, transcripts that
353 showed the highest levels of expression in sneaker males compared to other groups

354 were related to glutamate receptor genes, particularly AMPA ionotropic glutamate
355 receptors (Table S3, Supporting Information).
356
357 **Discussion**
358 Bluegill sunfish are a classic system for examining behavioral differences in
359 ARTs. In this study, we generated and assembled the first bluegill brain
360 transcriptome, and we identified candidate genes that contribute to differences in
361 male spawning tactics. The main differences in gene expression were found between
362 sneaker males when compared to the two other male tactics. Generally, sneaker
363 males showed higher expression in transcripts influencing neural activity, whereas
364 parental and satellite males exhibited higher expression in genes related to
365 translation and oxidoreductase activity.
366 One of our key findings is that a shared life history does not appear to be a
367 driving factor influencing similarity in gene expression in the brain of male tactics.
368 In bluegill, parental and cuckolder life histories are fixed, but within the cuckolder
369 life history, males transition from the sneaking to the satellite tactic as they age
370 (Gross 1982; Gross & Charnov 1991). Our data showed that, regardless of whether
371 comparisons were made across fixed (parental versus sneaker or parental versus
372 satellite) or plastic (sneaker versus satellite) tactics, sneaker males showed the
373 highest level of differentiation in gene transcription. Similar results have been
374 observed in the ocellated wrasse, *Symphodus ocellatus*, where sneaker males also
375 showed the greatest number of differentially expressed genes compared to nesting
376 and satellite males (Stiver *et al*. 2015). The expression differences in sneakers

377 compared to the other tactics in bluegill and the ocellated wrasse are likely partially
378 due to age because sneaker males are typically younger than satellite and parental
379 or territorial males. Indeed, a recent study in the short-lived fish *Nothobranchius*
380 *furzeri* found that genes related to translation elongation and ribosomal proteins are
381 up-regulated with age (Baumgart *et al*. 2014). Both translation elongation and
382 ribosomal proteins showed higher expression in parental and satellite males
383 compared to sneaker males in our data set. Additionally, the behaviors exhibited by
384 sneaker males during spawning differ in fundamental aspects from those of the
385 other male tactics. In the ocellated wrasse, for example, satellite and nesting males
386 cooperatively protect the nest from sneakers and other egg predators (Taborsky *et*
387 *al.* 1987); in bluegill, satellite and parental males associate closely with the female
388 throughout spawning, whereas sneakers dart in and out of the nest. These
389 differences in spawning tactics likely also contribute to the differences in gene
390 expression observed in the two studies. Thus, age and spawning tactic are
391 important contributors to gene expression patterns across ARTS, and life history is
392 not exclusively responsible for these differences.
393 Identifying distinct gene categories expressed by one ART type compared to
394 another provides information regarding the genes influencing behavioral
395 differences during spawning. Previous studies in sailfin mollies, *Poecilia latipinna,*
396 and Atlantic salmon, *Salmo salar*, indicate that sneaker males have increased
397 expression in genes related to neurotransmission and learning (Aubin-Horth *et al*.
398 2005; Fraser *et al*. 2014). We found that the GO terms consistently enriched in
399 bluegill sneaker males compared to both parental and satellite males were the

400 ionotropic glutamate signaling pathway and ionotropic gluatamate receptor activity.
401 Ionotropic gluatamate receptors are primarily excitatory neurotransmitter
402 receptors and play an important role in fast synaptic transmission (reviewed in
403 Lamprecht & LeDoux 2004). Two of these receptors, NMDA and AMPA, play
404 important roles in memory function and spatial learning (reviewed in Riedel *et al.*
405 2003). Blocking NMDA receptors impairs learning new spatial locations in goldfish
406 (Gómez *et al*. 2006). Futhermore, mice with impaired AMPA receptors, while
407 showing normal spatial learning, have impaired working spatial memory (i.e. their
408 ability to alter their spatial choice in response to changing environments is
409 impaired) (Reisel *et al*. 2002). We propose that increased expression of genes
410 related to spatial memory, particularly related to working spatial memory, could be
411 important for bluegill sneakers during spawning as they attempt to gain access to
412 nests while avoiding detection not only by the parental males, but also predators
413 that are common around the colony (Gross & MacMillian 1981). Bluegill sneakers
414 must also position themselves in close proximity to females so they can time sperm
415 release to coincide with female egg release (Stoltz & Neff 2006). Similarly, sailfin
416 molly sneakers, who also show enrichment in ionotropic glutamate related genes
417 (Fraser *et al*. 2014), probably benefit from increased working spatial memory
418 because they must successfully position themselves by the female for quick and
419 successful copulations. In this context, increased expression in gene pathways that
420 improve neural function related to working spatial memory are probably especially
421 beneficial to the sneaker tactic to increase their reproductive success.

422 There are a number of candidate genes that have been proposed to drive the
423 expression of alternative mating tactics (Schunter *et al*. 2015). In our study of
424 bluegill, we corroborate some of these candidates. For example, *cyp19a1b*, *epd*, and
425 *gal* had higher expression levels in parental males compared to sneaker males. The
426 expression patterns for all three genes are similar to what has been observed in
427 cichlids (Renn *et al*. 2008). In addition, expression of *epd* is lower in rainbow trout,
428 *Oncorhynchus mykiss*, males that use a sneaking tactic versus males that are
429 dominant and territorial (Sneedon *et al*. 2011), which is also consistent with our
430 findings. In contrast, the one candidate gene that responded opposite to
431 expectations was *egr1*. *Egr1* expression was lower in bluegill parental males
432 compared to sneaker or satellite males although previous work on cichlids found
433 that expression of this gene increases when subdominant males transition into
434 dominant males (Burmeister *et al*. 2005). However, *egr1* is an important
435 transcription factor involved in neural plasticity (Jones *et al*. 2001), so it may be
436 involved in regulating the switch from one tactic to another. Consequently, in
437 bluegill, this gene would be more important for individuals that alter their tactic
438 (sneaker to satellite) than for the fixed parental tactic. Taken together, our results
439 corroborate a role for *cyp19a1b*, *epd, gal, and egr1* as candidate genes contributing
440 to behavioral differences in ARTs across species.
441 We also found a transcript that may have a previously unrecognized function
442 in influencing male spawning behavior. Transcripts corresponding to splice variants
443 of cytosolic 5'-nucleotidase II (*nt5c2*) were significantly higher in parental males
444 when compared to all other male groups, including non-spawning males. The

445 protein product of *nt5c2* regulates purine metabolism (Bretonnet *et al*. 2005;
446 Walldén *et al*. 2007). Moreover, in the African cichlid, *A. burtoni*, uridine kinase
447 (*udk*) expression, a gene with similar function to *nt5c2*, is significantly higher in
448 dominant relative to subordinate males (Renn *et al*. 2008). While neither *nt5c2* nor
449 *udk* have been directly associated with spawning behavior in fishes, there is
450 evidence suggesting that altered expression levels of *nt5c2* in the brain can
451 significantly influence anxiety, mania, schizophrenia, and aggressive behaviors in
452 humans (Page *et al*. 2007), and altering levels of uridine in mice affects their level of
453 aggression toward an intruder (Kawasaki *et al*. 2013). Furthermore, in bluegill,
454 parental males display high levels of aggression to obtain nesting sites, circumvent
455 cuckoldry, and prevent egg predation by brood predators (Avila 1976; Colgan *et al*.
456 1979; Gross 1979; Gross & Macmillian 1981). The high levels of *nt5c2* expression in
457 spawning parental males suggests that this gene may have a role in influencing
458 parental male spawning behaviors in bluegill. Future work should examine how
459 *nt5c2* influences mating behaviors in the ARTs of this and other species.
460 In summary, our work describes differences in gene expression profiles in
461 the brains of bluegill male ARTs during spawning. The largest differences in
462 expression levels were observed when comparing sneakers to parental and satellite
463 males, suggesting that, in bluegill, tactic is more related to differences in gene
464 expression than is life history. Consistent with other studies, our work demonstrates
465 that sneaker males have greater expression of genes involved in neural function
466 relative to more territorial-type males, particularly in relation to working spatial
467 memory, as mediated by ionotropic glutamate receptors. We found support for the

468 previously identified candidate genes *cyp19a1b*, *epd, gal, and egr1* contributing to
469 behavioral differences in ARTs, but we also show evidence for a novel candidate
470 gene, *nt5c2,* implicated in these differences. We suggest that *nt5c2* may have a role
471 in mediating courtship or territorial behaviors within this species, and we
472 recommend that future work should characterize this gene further in other species.
473

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Author Contribution

C.G.P, R.K., and B.D.N. designed the experiment and collected samples. M.D.M. assembled the transcriptome. C.G.P. performed bioinformatic and statistical analyses and wrote the manuscript. All authors provided comments, contributed to the manuscript, and approved the final manuscript.

Data Accessibility

All raw sequence files are available on the Sequence Read Archive (SRA) through BioProject ID: PRJNA287763. Environmental data, RNA quality information, assembled transcriptome, the transcript count matrix, and R code for differential gene analysis are available on Dryad (http://dx.doi.org/10.5061/dryad.82fd8 and http://dx.doi.org/10.5061/dryad.10hh7).

Supporting Information

Table S1: Annotated Reference Transcriptome

Table S2: Transcripts with significantly higher expression in bluegill parental males compared to sneaker males.

Table S3: Transcripts with significantly higher expression in bluegill sneaker males compared to parental males.

Table S4: Biological Process and Molecular Function GO terms that are significantly enriched with genes differentially expressed between tactics

Table S5: Transcripts with significantly higher expression in bluegill parental males compared to satellite males.

Table S6: Transcripts with significantly higher expression in bluegill satellite males compared to parental males.

Table S7: Transcripts with significantly higher expression in bluegill satellite males compared to sneaker males.

Table S8: Transcripts with significantly higher expression in bluegill sneaker males compared to satellite males.

Table S9: Transcripts with significantly higher expression in spawning parental males compared to non-spawning parental males.

770 Table 1: Proposed candidate genes (Schunter *et al*. 2014) influencing teleost alternative reproductive
771 tactics (ARTs). POA = Pre-optic area

Proposed Candidate Genes	Function	Relationship to ARTs
Arginine Vasotocin (*avt*)	Non-mammalian homolog of vasopressin. Activates some aspects of sexual behavior	↑ in posterior POA of territorial cichlid males, but ↑ anterior POA of non-territorial (Greenwood *et al.* 2008); ↓ density of *avt* mRNA in POA in parental blenny males (Grober *et al.* 2002)
Gonadotrophin Releasing Hormone (*gnrh*)	Regulates release of lutenizing hormone and follicle-stimulating hormone from the pituitary gland	↑ in territorial cichlid males (Renn *et al*. 2008)
Cytochrome P450 family 19, subfamily A, polypeptide 1 (c*yp19a1*)	Brain aromatase. Key enzyme in estrogen biosynthesis	↑ in territorial cichlid males (Renn *et al*. 2008);↑ territorial blenny males (Gonçalves *et al*. 2008); ↑ territorial black-faced blenny males (Schunter *et al.* 2014); ↓ in the sonic motor nucleus of nesting type 1 (territorial) male plainfin midshipmen compared to type II (female mimic) males (Forlano *et al.* 2005)
Ependymin (*epd*)	Glycoprotein associated with neuroplasticity and neuronal regeneration. Also affects aggression levels in zebrafish (Sneddon *et al* 2011); associated with stress in trout (Thomson *et al.* 2011)	↑ in territorial cichlid males (Renn *et al.* 2008); ↓ in subordinate trout males (Sneddon *et al.* 2011)
Galanin/GMAP prepropeptide (*gal*)	Neuropeptide that influences neurotransmitters. Associated with sexual behaviors (Bloch *et al.* 1993), and parental care (Wu *et al.* 2014)	↑ in territorial cichlid males (Renn *et al.* 2008)
Somatostatin (*sst*)	Neuropeptide that regulates endocrine pathways. Also affects neurotransmitters	↑ in territorial blenny males (Schunter *et al.* 2014); ↑in territorial cichlid males (Renn *et al*. 2008)
Early growth response 1 (*egr1*)	Transcripton factor that influences neural plasticity	↑ when subdominant cichlid males switch to dominant (Burmeister *et al.* 2005)

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778 Table 2: Number of transcripts differentially expressed (DE) between each male bluegill tactic,
779 including the number of transcripts without Blastx hits, the number with unique Ensembl IDs, and
780 the number of transcripts assigned to specific GO terms

Comparisons	Num of DE Transcripts	Num of DE Transcripts without Blastx Hits	Num with Unique Ensembl Gene IDs	Num DE Gene IDs with GO Annotation
Parental vs Sneaker	9,279	516	5,396	2,430
Parental vs Satellite	1,141	82	879	317
Satellite vs Sneaker	2,590	184	1,852	351
Parental vs Non-Spawner	140	6	102	70

781

782 Table 3: Gene expression differences (Log2 fold change) among male tactics for proposed candidate
783 genes (see Table 1).

		Comparison between Male Tactics (Log2 Fold Change)			
Proposed Candidate Genes	**Isoform ID**	**Parent vs Sneak**	**Parent vs Sat**	**Sat vs Sneak**	**Non-Spawn vs Spawn Parent**
Arginine Vasotocin (*avt*)	c34708_g2_i1	0.45 [0.32]	-0.98 [0.09]	0.54 [0.33]	-0.74 [0.5]
Gonadotrophin Releasing Hormone *(gnrh*)	63124_g1_i1	0.76 [0.5]	0.32 [0.87]	0.44 [0.77]	-0.77 [0.88]
Cytochrome P450 19a 1b (c*yp19a1b*)	c48084_g2_i1	**0.93 [0.0002]**	0.64 [0.06]	0.28 [0.4]	-0.39 [0.58]
Ependymin *(epd*)	c44195_g1_i5	**1.54 [1.4 x 10^{-8}]**	0.66 [0.07]	**0.89 [0.007]**	0.51 [0.45]
Galanin/GMAP prepropeptide (*gal*)	c41071_g5_i2	**1.12 [0.0001]**	0.53 [0.91]	-0.59 [0.1]	-0.09 [0.97]
Somatostatin 1 *(sstr1*)	c3001_g1_i1	0.53 [0.15]	-0.39 [0.49]	**0.93 [0.03]**	-0.27 [0.88]
Somatostatin 3 (*sstr3*)	c46547_g6_i1	0.001 [1]	-0.25 [0.54]	0.25 [0.48]	-0.15 [0.9]
Early growth response 1 (*egr1*)	c37907_g1_i1	**-0.74 [0.02]**	**-0.91 [0.03]**	0.16 [0.72]	0.63 [0.42]

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785 Values in brackets represent p-values after false discovery rate correction. Values in bold are
786 significant at $p < 0.05$. Parent = parental male, Sneak = sneaker male, Sat = satellite male.
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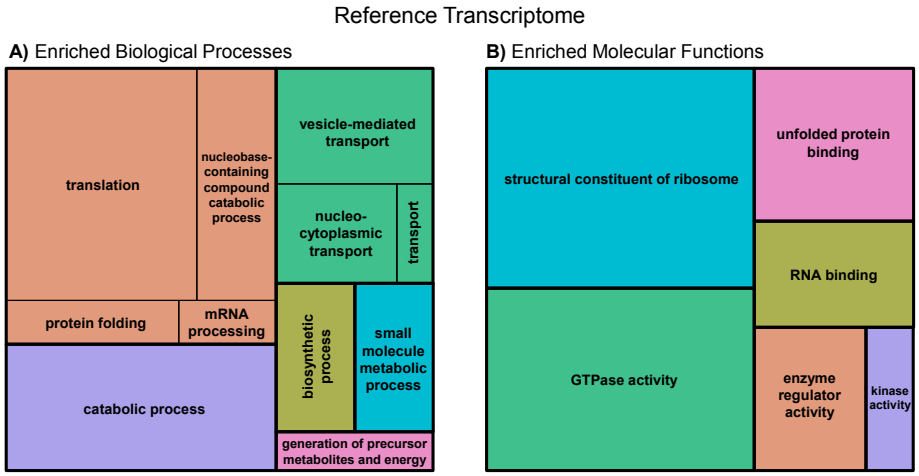

Fig. 1: GO terms related (A) biological processes and (B) molecular function that were significantly enriched in the bluegill reference transcriptome relative to the stickleback genome. Boxes of similar color can be grouped into the same GO term hierarchy. The size of each box reflects the –log10 p-value of the GO term.

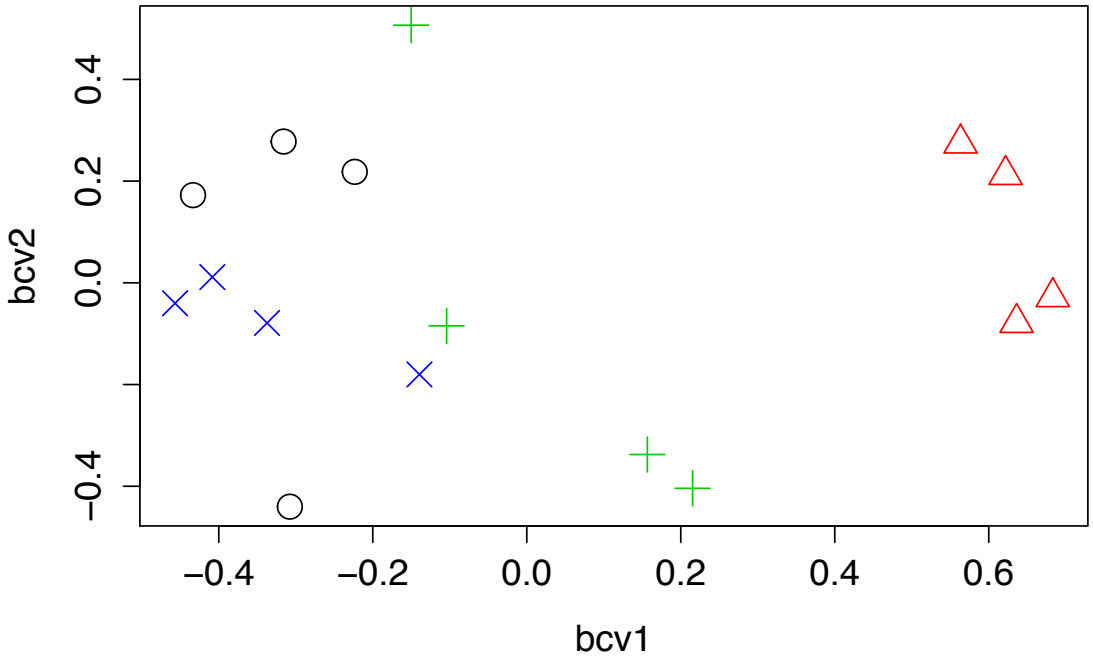

Fig. 2: Multi-dimensional space (MDS) plot based on the biological coefficient of variation (bcv) among bluegill male ARTs. Red triangles: sneaker males, green pluses: satellite males, black circles: spawning parental males, blue x: non-spawning parental males.

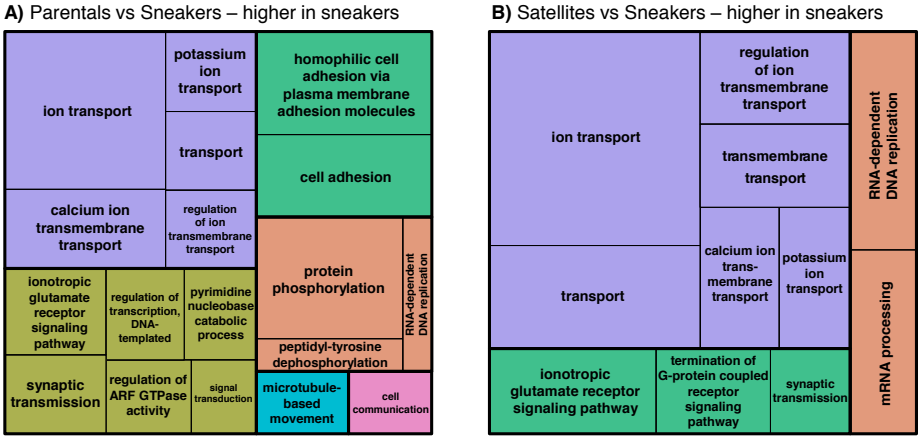

Figure 3: GO terms significantly enriched by genes with higher expression in sneaker males compared to (A) parental males and (B) satellite males. Boxes of similar color are grouped into the same GO term hierarchy. Box size reflects the –log10 p-value of the GO term.

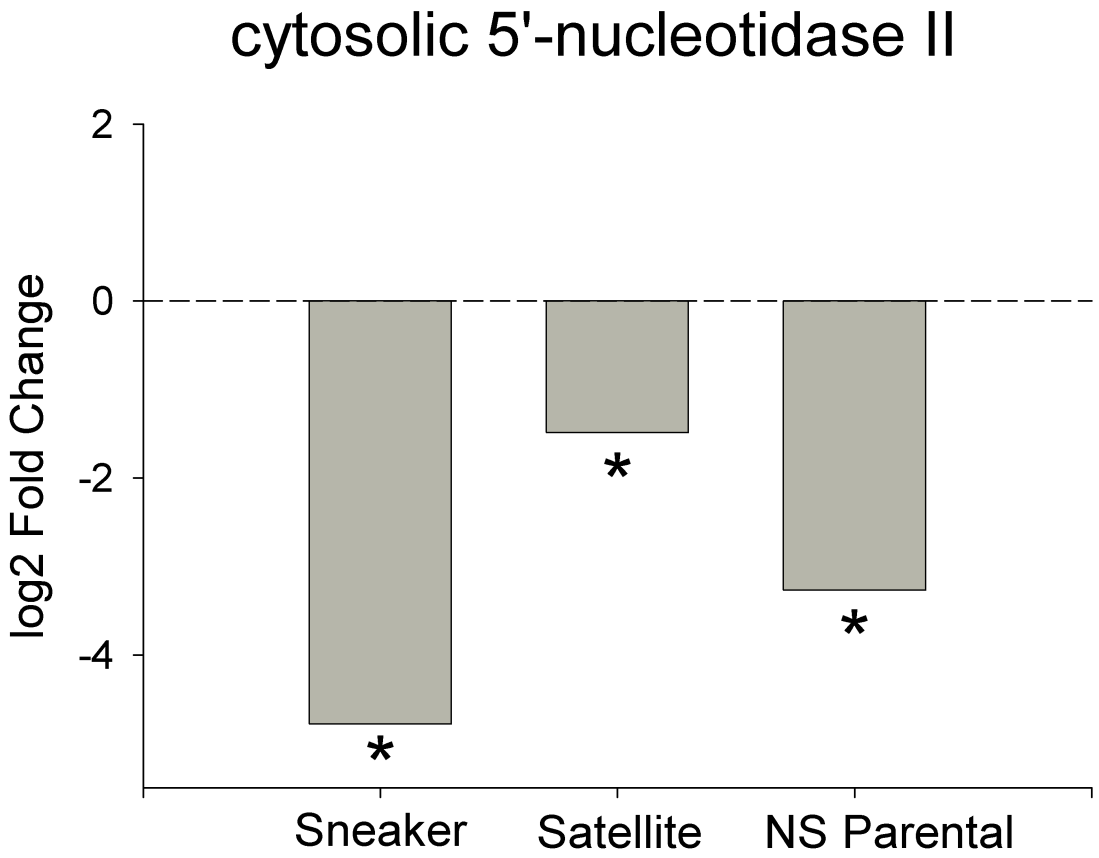

Figure 4: Log2 fold changes of sneaker, satellite, and non-spawning (NS) parental males relative to spawning parental males for cytosolic 5'-nucleotidase II (*nt5c2*). * indicates fold changes that are significantly different with p-values < 0.05 after FDR correction.