The neural basis of tadpole transport in poison frogs

Eva K. Fischer[1#], Alexandre Benoit-Roland[2#], Nora A. Moskowitz[1], Elicio E. Tapia[3], Kyle Summers[4], Luis A. Coloma[3], Lauren A. O'Connell[1*]

[1] Department of Biology, Stanford University, Stanford, California, United States of America

[2] Center for Systems Biology, Harvard University, Cambridge, Massachusetts, United States of America

[3] Centro Jambatu de Investigación y Conservación de Anfibios, Fundación Otonga, Quito, Ecuador

[4] Department of Biology, East Carolina University, Greenville, North Carolina, United States of America

[#] These authors contributed equally to this work

Running Title: Mechanisms of parental care in poison frogs

Word count: 5,286 (including references)

Key words: parental care, poison frog, phosphoTRAP, preoptic area, hippocampus, galanin

*To whom correspondence should be addressed:

Lauren A. O'Connell

Department of Biology

Stanford University

371 Serra Mall

Stanford, CA 94305

loconnel@stanford.edu

Abstract

Parental care has evolved repeatedly and independently across animals. While the ecological and evolutionary significance of parental behavior is well recognized, underlying mechanisms remain poorly understood. We took advantage of behavioral diversity across closely related species of South American poison frogs (Family Dendrobatidae) to identify neural correlates of parental behavior shared across sexes and species. We characterized differences in neural induction, gene expression in active neurons, and activity of specific neuronal types in three species with distinct parental care patterns: male uniparental, female uniparental, and biparental. We identified the medial pallium and preoptic area as core brain regions associated with parental care, independent of sex and species. Identification of neurons active during parental care confirms a role for neuropeptides associated with parental care in other vertebrates as well as identifying novel candidates. Our work highlights the potential for comparative, mechanistic studies to build a more complete understanding of how shared principles and species-specific diversity govern parental care and other social behavior.

Background

Parental care is an important adaptation that allows exploitation of novel habitats, influences fitness and survival of parents and offspring, and serves as an evolutionary precursor to other affiliative behavior [1,2]. Specialized parental care strategies have evolved repeatedly and independently across animals, yet the mechanisms underlying parental behavior and its evolution remain poorly understood. The neural mechanisms promoting parental care in females are best understood in mammals [3]; however, female uniparental care evolved at the base of the mammalian lineage and therefore provides limited clues to the evolutionary origins of parenting behavior. Moreover, studies of male parental care come mostly from biparental systems [4,5] in which parental behavior cannot easily be dissociated from pair bonding. What is needed to further understand the mechanisms underlying parental behavior and its evolution are comparative studies across closely-related species that vary in parental care strategies.

Parental care can be conceptualized as a complex set of inter-related behaviors controlled by brain regions involved in the integration of sensory, social, motivational, and cognitive aspects of care [6]. Across vertebrates, these functions are largely performed by the social decision-making network (SDMN; [7]), a highly interconnected group of evolutionarily ancient and functionally conserved brain regions. Although studies on the neural mechanisms of parental behavior are sparse outside mammals, and particularly lacking in amphibians and reptiles, the SDMN provides an ideal starting point for this work as network nodes and connectivity are well understood, highly conserved, and behaviorally important ligand/receptor complexes have been extensively studied.

Dendrobatid poison frogs show remarkable diversity in parental care across closely related species, including male uniparental care, female uniparental care, and biparental care. Parental care in poison frogs involves egg attendance during embryo development, generally followed by transportation of tadpoles “piggyback” to pools of water upon hatching [8–10]. In some species, mothers regularly return to nourish growing tadpoles with unfertilized, trophic eggs until

metamorphosis [9–11]. Importantly, both male and female care occur with and without pair bonding in this clade [12], allowing the dissociation of pair bonding from parental care. The diversity of behavioral care strategies between closely-related poison frog species affords a unique opportunity to identify physiological, neural, and molecular contributions to parental care and its evolution.

In the current study, we take advantage of three closely related focal species with distinct care patterns: *Dendrobates tinctorius* (male uniparental care), *Ranitomeya imitator* (biparental care), and *Oophaga sylvatica* (female uniparental). By comparing neural activity in parental frogs as well as their non-caregiving partners, we identify core brain regions active during tadpole transport independent of sex, species, and pair-bonding. To identify neuronal types mediating tadpole transport, we characterize gene expression and activity patterns specifically in behaviorally relevant neurons within core brain regions. Our experiments are the first to explore neural and molecular mechanisms of parental care in amphibians and demonstrate the utility of mechanistic studies in closely related, behaviorally distinct species in identifying core neural correlates of parental behavior.

Methods

Laboratory sample collection

Dendrobates tinctorius and *Ranitomeya imitator* frogs were housed in breeding pairs in the laboratory, allowing us to identify both parental individuals and their non-caregiving partners. To control for effects of experience, all pairs successfully reared at least one clutch from egg-laying through tadpole transport prior to the experiment. For the non-parental group, we collected frog pairs between parental bouts when they were not caring for eggs or tadpoles, collecting individuals of both the caregiving sex (non-transport; n=10 *D. tinctorius*, n=7 *R. imitator*) and their opposite sex partners (non-transport partner; n=9 *D. tinctorius*, n=8 *R. imitator*). For the tadpole

93 transport group, when we found transporting frogs, we collected both the tadpole transporting
94 individual (tadpole transporter; n=13 *D. tinctorius*, n=7 *R. imitator*) and its opposite sex, non-
95 transporting partner (transport partner; n=11 *D. tinctorius*, n=6 *R. imitator*). All brain tissue was
96 collected in an identical manner: frogs were captured, anesthetized with benzocaine gel, weighed
97 and measured, and euthanized by rapid decapitation. This entire process took less than 5
98 minutes. All procedures were approved by the Harvard University Animal Care and Use
99 Committee (protocol no. 12-10-1).
100
101 *Field sample collection*
102 *Oophaga sylvatica* (Puerto Quito-Santo Domingo population) were collected in field
103 enclosures in Ecuador in April and May of 2016. We collected non-parental control females (N=8)
104 from enclosures containing only mature females to ensure that frogs were not currently caring for
105 eggs or tadpoles. We collected tadpole transporting females (N=5) from enclosures containing
106 multiple males and females and therefore could not identify their non-caregiving male partners.
107 Frogs were captured, anesthetized with benzocaine gel, weighed and measured, and euthanized
108 by rapid decapitation. Procedures were approved by the Harvard University Animal Care and Use
109 Committee (protocol no. 15-03-239) and all samples were collected and imported in accordance
110 with Ecuadorian and US Law (collection permits: 005-15-IC-FAU-DNB/MA and 007-2016-IC-
111 FAU-DNB/MA; CITES export permit 16EC000007/VS issued by the Ministerio de Ambiente de
112 Ecuador).
113
114 *Immunohistochemistry*
115 Whole brains were placed into 4% paraformaldehyde at 4°C overnight and then
116 transferred to a 30% sucrose solution for cryoprotection. Once dehydrated, brains were
117 embedded in Tissue-Tek® O.C.T. Compound (Electron Microscopy Sciences, Hatfield, PA, USA),

118 rapidly frozen, and stored at -80°C until cryosectioning. We sectioned brains into four coronal
119 series at 14μm, allowed slides to dry completely, and stored slides at -80°C.

120 To assess the level of neural activity across brain regions, we used an antibody for
121 phosphorylated ribosomes (pS6; phospho-S6 Ser235/236; Cell Signaling, Danvers, MA, USA)
122 and followed standard immunohistochemical procedures for 3',3'-diaminobenzadine (DAB)
123 antibody staining (as in [13]). To ask whether neural activity was higher specifically in galanin
124 neurons, we combined the pS6 antibody with a custom-made galanin antibody (peptide
125 sequence: CGWTLNSAGYLLGPHAVDNHRSFNDKHGLA; Pocono Rabbit Farm & Laboratory,
126 Inc, Canadensis, PA, USA) and followed standard immunohistochemical procedures for
127 fluorescent double antibody labeling (as in [4]). Detailed methodological descriptions are in
128 Supplemental Materials.

129

130 *Microscopy and cell counts*

131 Stained brain sections were photographed on a Leica DMRE connected to a QImaging
132 Retiga 2000R camera at 20X magnification. We quantified labeled cells from photographs using
133 FIJI image analysis software [14]. Brain regions were identified using a custom dendrobatid frog
134 brain atlas (Supplemental Materials). We measured the area of candidate brain regions and
135 counted all labeled cells in a single hemisphere for each brain region across multiple sections.
136 We quantified cell number in the nucleus accumbens, the basolateral nucleus of the stria
137 terminalis, the habenula, the lateral septum, the magnocellular preoptic area, the medial pallium
138 (homolog of the mammalian hippocampus), the anterior preoptic area, the suprachiasmatic
139 nucleus, the striatum, the posterior tuberculum (homolog of the mammalian midbrain dopamine
140 cells representing the ventral tegmental area and substantia nigra), the ventral hypothalamus,
141 and the ventral pallium.

142 Fluorescently stained brain sections were photographed at 20X magnification on a Leica
143 DM4B compound microscope attached to a fluorescent light source. Each section was visualized

144 at three wavelengths (594nm, 488nm, 358nm) and images were pseudo-colored to reflect these
145 spectra. We used DAPI nuclear staining to identify brain regions as above and then quantified the
146 number of galanin positive cells, pS6 positive cells, and co-labeled cells from photographs of the
147 preoptic area using FIJI image analysis software [14]. We combined counts for all preoptic area
148 sub-regions due to the low overall number of galanin-positive neurons and because this more
149 closely reflected the neuroanatomical resolution of tissue punches used in phophoTRAP (see
150 below).
151
152 *Statistical analyses of cell counts*
153 We analyzed the relationship between parental behavior and pS6 neural activity to identify
154 brain regions whose activity differed during tadpole transport independent of sexes and species
155 (i.e. core parental care brain regions). We used generalized linear mixed models with a negative
156 binomial distribution appropriate for count data with unequal variances to test for differences in
157 pS6 positive cell number. For laboratory animals, behavioral group (tadpole transport vs non-
158 parental), sex, brain region, and their interactions were included as main effects predicting the
159 number of pS6-positive cells. For field sampled *O. sylvatica*, sex was omitted from the model as
160 we could not identify non-caregiving partners and collected only females. Individual was included
161 as a random effect, brain region area as a covariate to control for body size differences between
162 frogs, known size differences between brain regions, and rostral to caudal size/shape variation
163 within brain regions. We explored main effects of group, sex, and regional differences in further
164 detail using *post hoc* comparisons Tukey adjusted for multiple hypothesis testing.
165 We tested for differences in the number and activity of galanin neurons using generalized
166 linear mixed models. To compare the number of galanin neurons, we included behavioral group
167 (tadpole transport vs non-parental), sex, and their interactions as main effects predicting the
168 number of galanin positive cells using a negative binomial distribution appropriate for count data
169 with unequal variances. To analyze activity differences in preoptic area galanin neurons, we

170 included behavioral group, sex, and their interactions as main effects predicting the proportion of
171 pS6 positive galanin (i.e. co-labeled) cells using a binomial distribution. All analyses were
172 performed separately for each species using SAS Statistical Software (SAS 9.4; SAS Institute for
173 Advanced Analytics).
174
175 *PhosphoTRAP library construction & sequencing*
176 We collected *D. tinctorius* males that were found transporting tadpoles to males that
177 currently had tadpoles present in the leaf litter but had not yet transported them. Males were
178 sacrificed as described above (N=9 per group). Brains were removed, embedded in Tissue-Tek®
179 O.C.T. Compound, frozen on dry ice, and stored at -80°C for no more than 1 month. Once all
180 animals had been collected, brains were sectioned at 100 µm on a cryostat and thaw mounted
181 on SuperFrost Plus slides. A 0.96 mm tissue micro punch tool was used to isolate the medial
182 pallium and rostral hypothalamus (anterior, medial, and magnocellular preoptic area and
183 suprachiasmatic nucleus). To provide enough starting material for PhosphoTRAP, brain regions
184 from three individuals were combined into a single sample, for a total of three biological replicates
185 per group. PhosphoTRAP libraries for total (TOT) and immunoprecipitated (IP) RNA from each
186 sample were constructed following [15] (details in Supplemental Materials). Libraries were then
187 pooled in equimolar amounts and sequenced on an Illumina HiSeq 2500.
188
189 *PhosphoTRAP analysis*
190 To analyze phosphoTRAP data we first quantified gene expression by mapping
191 sequenced reads back to a brain tissue specific *D. tinctorius* transcriptome (Fischer & O'Connell,
192 *unpublished*) and estimated their abundance using Kallisto [16]. As gene expression is known to
193 differ across brain regions [17], we performed all subsequent analysis steps separately for the Mp
194 and POA. Analysis methods are described in detail in the Supplemental Materials. Briefly, we
195 normalized read counts using DESeq2 [18] and quantified transcript enrichment/depletion in

active neurons as a log-fold difference between transcript counts from immunoprecipitated (IP) and total (TOT) mRNA for each sample. We then calculated differential fold enrichment between parental and non-parental individuals by dividing mean log-fold expression values from the two behavioral groups. We refer to this final metric as the log-fold difference ratio between tadpole transport and non-transport behavioral groups.

Our primary objective was to utilize phosphoTRAP data to identify Mp and POA cell types whose activity differed between tadpole transport and non-parental individuals. To this end, we restricted further analysis to a subset of 158 transcripts representing cell types with known roles in parental care (Table S1). We identified transcripts as significantly enriched/depleted based on a combination of log-fold enrichment thresholds (>4) and permutation testing (Supplemental Materials). Permutation testing and visualization were done using R Statistical Software (version 3.5.0; the R Foundation for Statistical Computing).

Results

Neural induction during tadpole transport

We compared neural activity patterns in tadpole transporters and their non-transporting partners across three closely related poison frog species with distinct parental care strategies (Fig. 1A). Differences in neural activity depended on behavioral group, sex, and brain region (Fig. 1B; Table 1) and associations between behavioral group and neural induction were brain region specific (Table 1; group*region: *D. tinctorius*: $F_{1,2515}$=5.00, p<0.0001; *R. imitator*: $F_{12,557}$=6.85, p<0.0001; *O. sylvatica*: $F_{12,557}$=5.53, p<0.0001). We found overall differences between the transporting and non-transporting sex in male uniparental *D. tinctorius* (sex*group*region: $F_{1,2515}$=3.89, p<0.0001) but not biparental *R. imitator* (Table 1; Fig. 1B). Indeed, *post hoc* analyses of region-specific differences revealed greater similarity between sexes in biparental and monogamous *R. imitator* than male uniparental *D. tinctorius* (Fig. 1B,C; Table S2).

222 Comparing neural activity patterns associated with parental care across species allowed
223 us to identify brain regions important in parental care independent of sex and species (i.e. core
224 parental care brain regions). We observed parallel increases in neural activity in tadpole
225 transporting individuals in two core brain regions across all species: the preoptic area (POA) and
226 the medial pallium (Mp; homolog of the mammalian hippocampus. In the POA, patterns differed
227 by subdivision, with female-specific effects in the magnocellular POA and male-specific effects in
228 the anterior POA (Fig. 1C). We also observed increased neural activity in the Mp of the non-
229 caregiving partners of tadpole-transporters (Fig. 1C).
230
231 *Gene expression in behaviorally relevant neurons*
232 Following identification of core brain regions active during tadpole transport across
233 species and sexes, we sought to identify behaviorally relevant neuronal types within these
234 regions. We found 25 transcripts with significant log-fold expression enrichment/depletion in the
235 POA and 32 transcripts with significant log-fold expression enrichment/depletion in the Mp, seven
236 of which were overlapping between brain regions (Fig. 2; Table S3). Of the overlapping
237 transcripts, four had log-fold expression differences in the same direction (galanin, prolactin
238 receptor, neuropeptide Y receptor 2, brain specific angiogenesis inhibitor associated protein 2)
239 and three had log-fold expression differences in opposite directions (aquaporin 4, dopamine
240 receptor 1B, leptin receptor) between brain regions (Fig. 2).
241
242 *Galanin neuron number and activity*
243 Recent demonstrations of the importance of galanin in mediating parental care in mice
244 [19,20] and the enrichment of galanin transcripts in neurons active during tadpole transport led
245 us to ask whether activity differences specifically in POA galanin neurons were associated with
246 parental care. Parental *R. imitator* had significantly more galanin neurons than did non-parental
247 *R. imitator,* independent of sex (behavioral group: $F_{1,404}$=4.58, p=0.0329), but there were no

248 differences in galanin neuron number in *D. tinctorius* or *O. sylvatica* (Fig. S2). Both *D. tinctorius*
249 and *R. imitator* showed differences in galanin neuron activity associated with parental care, but
250 not in the same manner: in *D. tinctorius* the proportion of active galanin neurons was greater in
251 the female partners of non-transport males than any other group (sex*behavioral group:
252 $F_{1,40}$=12.73, p=0.0010; Fig. 3). In contrast, in *R. imitator* the proportion of active galanin neurons
253 was greater during tadpole transport in both males and females (behavioral group: $F_{1,26}$=8.15,
254 p=0.0083; Fig. 3). We observed no differences in the proportion of active galanin neurons
255 between tadpole-transporting and non-transporting *O. sylvatica* females.
256
257
258 **Discussion**
259 Parental care requires the coordination of hormonal, neural, and molecular changes, many
260 of which remain poorly understood. We took advantage of shared parental behavior across three
261 poison frog species with distinct parental care strategies, combining lab and field data to
262 disentangle sex- and species- specific mechanisms from core neural mechanisms at the levels of
263 brain regions, gene expression, and neuron type. We identified the medial pallium and preoptic
264 area as core brain regions associated with parental care and demonstrated expression changes
265 in genes associated with parental care in other vertebrates. Mechanistic studies in closely related,
266 behaviorally variable poison frogs offer a unique opportunity to distinguish shared principles and
267 neural diversity in the mechanisms mediating the maintenance and evolution of parental care.
268
269 *Core brain regions for parental care*
270 By comparing patterns of neural activity across closely related species with distinct
271 parental care strategies, we were able to identify core brain regions in which increased neural
272 induction during parental care was sex and species independent. We observed increased neural
273 induction in the medial pallium (Mp) and one or more subdivisions of the preoptic area (POA)

274 during parental care in all focal species. The POA's widespread connections with other brain
275 regions and high density of neuromodulators make it ideally positioned to modulate complex
276 social behavior, including parental care. Although data outside mammals is sparse, POA activity
277 is associated with parental behavior across vertebrates, including mammals [3], birds [3,21], fish
278 [22], and now frogs. In brief, the POA appears to be a core node in parental care circuitry across
279 vertebrates. Importantly, parental care has evolved independently across these clades, indicating
280 convergence across behavioral and neural levels.

281 In contrast with the POA, the Mp is not commonly associated with parental care. Although
282 the precise function of the hippocampus and its non-mammalian homologs remains an area of
283 active research, this brain region is classically implicated in memory, and specifically spatial
284 memory [23,24]. Poison frogs inhabit complex rain forest environments in which tadpole
285 deposition sites are a limited resource of variable quality. Behavioral studies in poison frogs
286 document spatial learning abilities [25] and demonstrate the importance of spatial memory for
287 navigating back to high-quality tadpole deposition pools [26] and for relocating offspring in egg
288 provisioning species [27]. Increased neural induction in the Mp during tadpole transport is
289 therefore in line with the unique ecological and evolutionary pressures associated with parental
290 care in poison frogs. Indeed, spatial cognition is an important, but rarely examined, component of
291 parental care [28,29], and comparisons of hippocampal involvement in parental care across
292 species may yield interesting results given the functional conservation of this structure across
293 vertebrates [30].

294

295 *Shared parental care circuitry across sexes*

296 The strength of our comparative design is highlighted by identification of inter-specific
297 neural activity patterns between sexes. Neural activity during tadpole transport differed between
298 males and females in uniparental *D. tinctorius*, but not biparental *R. imitator*. Females are not
299 directly involved in tadpole transport in either species; however, biparental *R. imitator* females

300 provide parental care in the form of egg attendance prior to tadpole transport and tadpole
301 provisioning following transport [12,30,31]. Thus, similar patterns of neural activity in male and
302 female biparental *R. imitator* could arise either because both sexes are in a "parental state" that
303 modulates long-term circuit activity or because even indirect involvement in tadpole transport
304 activates parental circuitry (i.e. female frogs have to know where their tadpoles are transported in
305 order to return to feed them). In either case, similarities in neural activity patterns associated with
306 parental care across sexes suggest that parental care circuitry is conserved across sexes.

307 In addition to broad sex similarities in *R. imitator*, we also observed increased neural
308 activity in the Mp of non-caregiving *D. tinctorius* females. While they are not the typically
309 caregiving sex, females of *D. tinctorius* and related species will occasionally perform tadpole
310 transport [32,33]. This behavioral flexibility demonstrates that parental circuits are present and
311 can be activated under certain circumstances in females, and we suggest an increase in Mp
312 neural activity is related to females' monitoring of their partners' behavior and ability to perform
313 tadpole transport in the absence of their male partners. In other words, females may monitor male
314 behavior in order to assess when and if they need to take over parental behaviors to ensure the
315 survival of their offspring. The diversity of behavioral care strategies between species combined
316 with this behavioral flexibility within species in poison frogs affords a unique opportunity to further
317 disentangle the evolution of sex-specific parental care circuits in future.

318

319 *Expression variation in behaviorally relevant neurons*

320 Using *D. tinctorius* males, we characterized gene expression differences specifically in
321 neurons active within the POA and Mp during parental care, focusing our analyses on genes
322 previously identified as markers of neuronal types involved in parental care [34]. Of particular
323 interest in the POA were increased expression of the vasopressin 1b receptor, a gonadotropin-
324 releasing hormone receptor, and a number of stress response related genes (Urocortin-3, CART,
325 CRF binding protein). Links between vasopressin and parental care have been demonstrated in

326 rodents [35,36] and vasopressin and gonadotropin releasing hormone may also influence parental
327 care indirectly through their regulation of other molecules with known roles in parental care (e.g.
328 oxytocin, prolactin) [3]. Stress hormones are known to increase in response to the behavioral and
329 metabolic demands of parental care [37,38] providing a link between parental behavior and the
330 observed upregulation of stress-related signaling pathways.

331 Notable in the Mp were increased expression of vasopressin and androgen receptor
332 transcripts. As described above, vasopressin signaling is widely implicated in parental care, and
333 has been specifically linked to space use and behavioral and life-history trade-offs in parental
334 prairie voles [28,29]. Space use and navigational abilities differ between males and females in
335 many species, and it has been proposed that greater navigational abilities in males are a side
336 effect of increased androgen signaling [39]. Increased androgen signaling during parental care in
337 poison frogs could facilitate the heightened spatial cognition important during tadpole transport.
338 Increasing signaling via region specific receptor expression overcomes the lower testosterone
339 levels typically observed in parental males [40].

340 In addition to changes specific to either the POA or Mp, we observed a number of
341 transcripts with significant expression differences in both regions. Among them were dopamine
342 and prolactin receptors, and a number of molecules and receptors most commonly implicated in
343 feeding behavior (galanin, leptin receptor, NPY receptor). Dopamine and prolactin play known
344 roles in parental care [40–42], while other shared transcripts (and some of those unique to a single
345 brain region) are traditionally associated with feeding behavior. There is growing recognition that
346 molecules traditionally classified as feeding-related play important roles in mediating social
347 behavior, providing exciting opportunities to explore the repeated targeting of feeding related
348 mechanisms in the convergent evolution of parental care [43].

349

350 *Galanin and parental care*

351 Initially described in relation to feeding behavior, recent work uncovered a role for POA
352 galanin neurons in driving parental care in both male and female mice [19,20]. We found a positive
353 association between parental care and galanin neuron number and activity in biparental *R.*
354 *imitator*, but not in male uniparental *D. tinctorius,* nor female uniparental *O. sylvatica*. Indeed, the
355 only significant difference outside *R. imitator*, was a relative increase in galanin neuron activity in
356 the female partners of non-transporting male *D. tinctorius*, and we note that the percent of active
357 galanin neurons was overall low in all species.

358 While recent work demonstrates a sex-independent, behavior-specific link between
359 galanin neuron activity and parental care [19,20], the earliest work on POA galanin in rodents
360 showed that microinjection of galanin into the POA of male rats facilitated copulatory behavior
361 [44], and work in fish similarly suggests an association between male courtship behavior and
362 galanin signaling [45,46]. Thus, species in which the role of galanin in social behavior has been
363 explored vary in parental care strategy: rats are female uniparental, only some male mice exhibit
364 male care, and fish include both male uniparental and female uniparental species. Together with
365 our findings across frog species with distinct care patterns, these observations suggest that the
366 role of galanin signaling in parental care may be mediated – both acutely and evolutionarily – by
367 life history differences related to parental care, interactions among partners, and male courtship
368 strategy. In brief, galanin appears to have been repeatedly evolutionarily co-opted to modulate
369 social behavior, but the type(s) of social behavior influenced by galanin signaling are complex,
370 mediated by the behavioral variation and evolution history, and providing fertile ground for future
371 comparative research.

372

373 *Conclusions*

374 Our findings lay the foundation for exciting work using poison frogs as a model to explore
375 neural and molecular mechanisms of parental care, sex-specific behavioral patterns, and the
376 integration of social and environmental cues to coordinate complex social behavior. We identified

core brain regions associated with tadpole transport across dendrobatid poison frogs with distinct care strategies. Moreover, we confirmed a role in amphibians for hormones and neuropeptides associated with parental care in other vertebrates. While increased POA activity was associated with parental care across species, activity specifically of galanin neurons differed between species, suggesting that shared brain regions may nonetheless rely on unique neuronal types to mediate similar behavior. Comparative studies in closely related, but behaviorally distinct, species provide opportunities to build a more holistic understanding of how shared principles and species-specific diversity govern parental care.

Acknowledgements

We thank the O'Connell Lab frog caretakers for help with animal care, Lola Guarderas (Wikiri) and Manuel Morales-Mite (Centro Jambatu) for field work support, and Julie Butler, Hans Hofmann and the members of the O'Connell Lab for comments on previous versions of the manuscript.

Funding

We gratefully acknowledge support from a Harvard University Bauer Fellowship, the International Society for Neuroethology Konishi Research Award, and the Graduate Women in Science Adele Lewis Grant Fellowship to LAO, and a postdoctoral fellowship (NSF-1608997) to EKF. LAC and EET were supported by Wikiri and the Saint Louis Zoo to Centro Jambatu.

Data availability

Cell counts, read counts from phosphoTRAP, R code for phosphoTRAP analysis, and the *D. tinctorius* brain atlas are available as Supplemental Materials associated with the manuscript. Raw sequencing reads will be made available through the NCBI SRA repository upon publication.

403

404 **Authors' contributions**

405 LAO conceived of the study; LAO, KS and LAC designed and coordinated the study; EKF,
406 ABR, NAM, and EET collected samples; EKF and LAO performed molecular work and data
407 analysis; EKF and LAO wrote the manuscript with input from all authors; All authors gave final
408 approval for publication and agree to be held accountable for the work performed therein.

409 **References**

410 1. Clutton-Brock TH. 1991 *The Evolution of Parental Care*. Princeton University Press.

411 2. Royle NJ, Smiseth PT, Kölliker M. 2012 *The Evolution of Parental Care*. Oxford University
412 Press.

413 3. Numan M, Insel TR. 2006 *The Neurobiology of Parental Behavior*. Springer Science &
414 Business Media.

415 4. O'Connell LA, Matthews BJ, Hofmann HA. 2012 Isotocin regulates paternal care in a
416 monogamous cichlid fish. *Hormones and Behavior*. **61**, 725–733.
417 (doi:10.1016/j.yhbeh.2012.03.009)

418 5. Kirkpatrick B, Kim JW, Insel TR. 1994 Limbic system fos expression associated with
419 paternal behavior. *Brain Res.* **658**, 112–118.

420 6. Pereira M, Ferreira A. 2016 Neuroanatomical and neurochemical basis of parenting:
421 Dynamic coordination of motivational, affective and cognitive processes. *Hormones and*
422 *Behavior*. **77**, 72–85. (doi:10.1016/j.yhbeh.2015.08.005)

423 7. O'Connell LA, Hofmann HA. 2012 Evolution of a Vertebrate Social Decision-Making
424 Network. *Science*. **336**, 1154–1157. (doi:10.1126/science.1218889)

425 8. Brown JL, Morales V, Summers K. 2010 A key ecological trait drove the evolution of
426 biparental care and monogamy in an amphibian. *Am. Nat.* **175**, 436–446.

427 9. Pröhl H, Hödl W. 1999 Parental investment, potential reproductive rates, and mating
428 system in the strawberry dart-poison frog, *Dendrobates pumilio*. *Behavioral Ecology and*
429 *Sociobiology*. **46**, 215–220. (doi:10.1007/s002650050612)

430 10. Weygoldt P. 2009 Evolution of parental care in dart poison frogs (Amphibia: Anura:
431 Dendrobatidae). *Journal of Zoological Systematics and Evolutionary Research*. **25**, 51–67.
432 (doi:10.1111/j.1439-0469.1987.tb00913.x)

433 11. Summers K, Earn DJD. 1999 The cost of polygyny and the evolution of female care in
434 poison frogs. *Biological Journal of the Linnean Society*. **66**, 515–538. (doi:10.1111/j.1095-
435 8312.1999.tb01924.x)

436 12. Summers K, Tumulty J. 2014 Parental Care, Sexual Selection, and Mating Systems in
437 Neotropical Poison Frogs. *Sexual Selection*. 289–320. (doi:10.1016/b978-0-12-416028-
438 6.00011-6)

439 13. Fischer EK, Westrick SE, Hartsough L, Hoke KL. 2018 Differences in neural activity, but not
440 behavior, across social contexts in guppies, *Poecilia reticulata*. *Behavioral Ecology and*
441 *Sociobiology*. **72**. (doi:10.1007/s00265-018-2548-9)

442 14. Schindelin J *et al.* 2012 Fiji: an open-source platform for biological-image analysis. *Nat.*
443 *Methods* **9**, 676–682.

444 15. Knight ZA, Tan K, Birsoy K, Schmidt S, Garrison JL, Wysocki RW, Emiliano A, Ekstrand MI,
445 Friedman JM. 2012 Molecular profiling of activated neurons by phosphorylated ribosome
446 capture. *Cell* **151**, 1126–1137.

447 16. Bray NL, Pimentel H, Melsted P, Pachter L. 2016 Near-optimal probabilistic RNA-seq
448 quantification. *Nat. Biotechnol.* **34**, 525–527.

449 17. Lein ES *et al.* 2007 Genome-wide atlas of gene expression in the adult mouse brain.
450 *Nature* **445**, 168–176.

451 18. Love MI, Huber W, Anders S. 2014 Moderated estimation of fold change and dispersion for

452 RNA-seq data with DESeq2. *Genome Biol.* **15**, 550.

453 19. Wu Z, Autry AE, Bergan JF, Watabe-Uchida M, Dulac CG. 2014 Galanin neurons in the
454 medial preoptic area govern parental behaviour. *Nature* **509**, 325–330.

455 20. Kohl J *et al.* 2018 Functional circuit architecture underlying parental behaviour. *Nature* **556**,
456 326–331.

457 21. Ruscio MG, Adkins-Regan E. 2004 Immediate early gene expression associated with
458 induction of brooding behavior in Japanese quail. *Horm. Behav.* **46**, 19–29.

459 22. Demski LS, Knigge KM. 1971 The telencephalon and hypothalamus of the bluegill
460 (Lepomis macrochirus): evoked feeding, aggressive and reproductive behavior with
461 representative frontal sections. *J. Comp. Neurol.* **143**, 1–16.

462 23. Rodríguez F, López JC, Vargas JP, Gómez Y, Broglio C, Salas C. 2002 Conservation of
463 spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. *J. Neurosci.*
464 **22**, 2894–2903.

465 24. Thompson RF. 1986 The neurobiology of learning and memory. *Science* **233**, 941–947.

466 25. Liu Y, Day LB, Summers K, Burmeister SS. (*in press*) A cognitive map in a poison frog. *J.*
467 *Expt. Biol*.

468 26. Pašukonis A, Ringler M, Brandl HB, Mangione R, Ringler E, Hödl W. 2013 The Homing
469 Frog: High Homing Performance in a Territorial Dendrobatid Frog (Dendrobatidae).
470 *Ethology* **119**, 762–768.

471 27. Stynoski JL. 2009 Discrimination of offspring by indirect recognition in an egg-feeding
472 dendrobatid frog, *Oophaga pumilio*. *Animal Behaviour*. **78**, 1351–1356.
473 (doi:10.1016/j.anbehav.2009.09.002)

474 28. Okhovat M, Berrio A, Wallace G, Ophir AG, Phelps SM. 2015 Sexual fidelity trade-offs
475 promote regulatory variation in the prairie vole brain. *Science* **350**, 1371–1374.

476 29. Ophir AG, Wolff JO, Phelps SM. 2008 Variation in neural V1aR predicts sexual fidelity and
477 space use among male prairie voles in semi-natural settings. *PNAS* **105**, 1249–1254.

478 30. Butler AB. 2017 Of Horse-Caterpillars and Homologies: Evolution of the Hippocampus and
479 Its Name. *Brain Behav. Evol.* **90**, 7–14.

480 31. Brown JL, Morales V, Summers K. 2008 Divergence in parental care, habitat selection and
481 larval life history between two species of Peruvian poison frogs: an experimental analysis.
482 *J. Evol. Biol.* **21**, 1534–1543.

483 32. Ringler E, Pašukonis A, Hödl W, Ringler M. 2013 Tadpole transport logistics in a
484 Neotropical poison frog: indications for strategic planning and adaptive plasticity in anuran
485 parental care. *Front. Zool.* **10**, 67.

486 33. Tumulty J, Morales V, Summers K. 2014 The biparental care hypothesis for the evolution of
487 monogamy: experimental evidence in an amphibian. *Behavioral Ecology*. **25**, 262–270.
488 (doi:10.1093/beheco/art116)

489 34. Moffitt JR *et al.* 2018 Molecular, spatial, and functional single-cell profiling of the
490 hypothalamic preoptic region. *Science* **362**. (doi:10.1126/science.aau5324)

491 35. Bester-Meredith JK, Marler CA. 2003 Vasopressin and the transmission of paternal
492 behavior across generations in mated, cross-fostered Peromyscus mice. *Behav. Neurosci.*
493 **117**, 455–463.

494 36. Rilling JK, Mascaro JS. 2017 The neurobiology of fatherhood. *Curr Opin Psychol* **15**, 26–
495 32.

496 37. Jeffrey JD, Cooke SJ, Gilmour KM. 2014 Regulation of hypothalamic-pituitary-interrenal
497 axis function in male smallmouth bass (*Micropterus dolomieu*) during parental care. *Gen.*
498 *Comp. Endocrinol.* **204**, 195–202.

499 38. O'Connor CM, Gilmour KM, Arlinghaus R, Van Der Kraak G, Cooke SJ. 2009 Stress and
500 Parental Care in a Wild Teleost Fish: Insights from Exogenous Supraphysiological Cortisol
501 Implants. *Physiological and Biochemical Zoology*. **82**, 709–719. (doi:10.1086/605914)

502 39. Clint EK, Sober E, Garland T Jr, Rhodes JS. 2012 Male superiority in spatial navigation:
503 adaptation or side effect? *Q. Rev. Biol.* **87**, 289–313.

504 40. Adkins-Regan E. 2013 Hormones and Animal Social Behavior. Princeton University Press.

505 41. Angelier F, Wingfield JC, Tartu S, Chastel O. 2016 Does prolactin mediate parental and
506 life-history decisions in response to environmental conditions in birds? A review. *Horm.*
507 *Behav.* **77**, 18–29.

508 42. Schradin C, Anzenberger G. 1999 Prolactin, the Hormone of Paternity. *News Physiol. Sci.*
509 **14**, 223–231.

510 43. Fischer EK, O'Connell LA. 2017 Modification of feeding circuits in the evolution of social
511 behavior. *The Journal of Experimental Biology*. **220**, 92–102. (doi:10.1242/jeb.143859)

512 44. Bloch GJ, Butler PC, Kohlert JG, Bloch DA. 1993 Microinjection of galanin into the medial
513 preoptic nucleus facilitates copulatory behavior in the male rat. *Physiol. Behav.* **54**, 615–
514 624.

515 45. Partridge CG, MacManes MD, Knapp R, Neff BD. 2016 Brain Transcriptional Profiles of
516 Male Alternative Reproductive Tactics and Females in Bluegill Sunfish. *PLoS One* **11**,
517 e0167509.

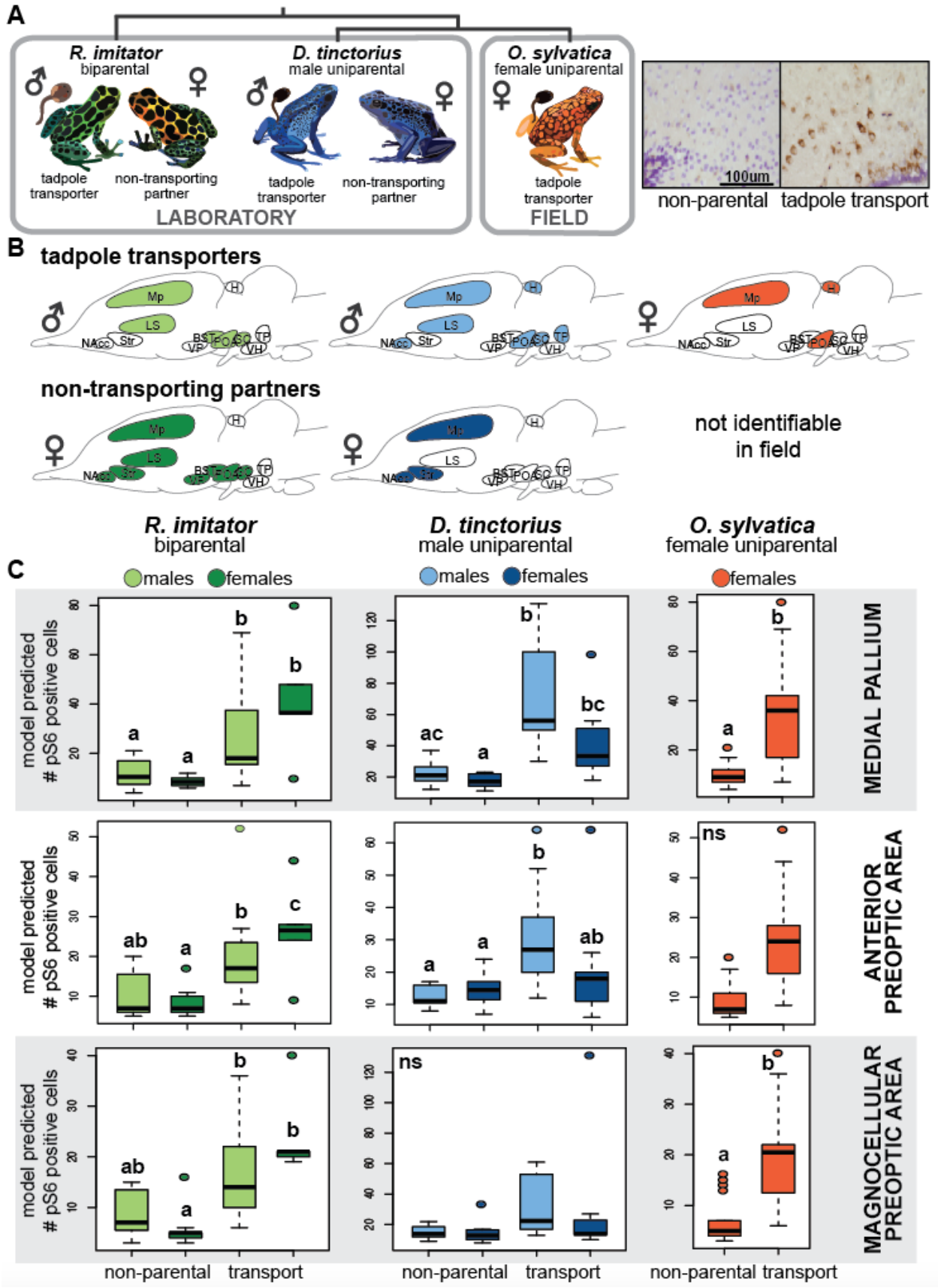
518 46. Tripp JA, Feng NY, Bass AH. 2018 Behavioural tactic predicts preoptic-hypothalamic gene
519 expression more strongly than developmental morph in fish with alternative reproductive
520 tactics. *Proc. Biol. Sci.* **285**. (doi:10.1098/rspb.2017.2742)

521 **Tables & Figures**
522
523

Table 1. Summary of main statistical effects for neural induction differences.

		df	F value	p value
R. imitator	group	1,1731	13.83	0.0002
	sex	1,1731	0.64	0.4242
	region	12,1731	87.48	<0.0001
	sex*group	1,1731	1.25	0.2630
	group*region	12,1731	6.85	<0.0001
	sex*region	12,1731	2.69	0.0013
	sex*group*region	12,1731	1.45	0.1344
D. tinctorius	group	1,2519	9.73	0.0018
	sex	1,2519	1.36	0.2443
	region	12,2519	80.15	<0.0001
	sex*group	1,2519	1.76	0.1844
	group*region	12,2519	5.00	<0.0001
	sex*region	12,2519	3.39	<0.0001
	sex*group*region	12,2519	3.89	<0.0001
O. sylvatica	group	1,557	1.02	0.3126
	region	12,557	9.40	<0.0001
	group*region	12,557	5.53	<0.0001

524


A
R. imitator
biparental
D. tinctorius
male uniparental
O. sylvatica
female uniparental
tadpole transporter
non-transporting partner
tadpole transporter
non-transporting partner
tadpole transporter
LABORATORY
FIELD
100um
non-parental
tadpole transport
B
tadpole transporters
non-transporting partners
not identifiable in field
Mp
LS
NAcc
Str
BST
POA
SO
TP
VP
VH
H
C
males
females
model predicted # pS6 positive cells
MEDIAL PALLIUM
ANTERIOR PREOPTIC AREA
MAGNOCELLULAR PREOPTIC AREA
ns
non-parental
transport

525

Figure 1. Patterns of neural induction associated with parental care. (A) Overview of experimental design. Our comparative approach allowed us to identify brain regions important in parental care independent of sex and species. **(B)** Overview of brain regions showing differences in neural activity between parental and non-parental individuals (shaded) for tadpole transporting sex and their non-transporting partners. Small symbols indicate the sex of transporting and non-transporting partner individuals. Comparing across species, we identified Mp and POA as active during tadpole transport regardless of sex and species (i.e. as core parental care brain regions). **(C)** Detailed results for core brain regions. Letters above the box plots indicate significant group differences ($p<0.05$). Representative micrographs of pS6 staining (brown) with cresyl violet nuclear stain (purple) from the mPOA are shown at top right. Abbreviations: BST = basolateral nucleus of the stria terminalis, H = habenula, Ls = lateral septum, Mp = medial pallium (homolog of the mammalian hippocampus), NAcc = nucleus accumbens, aPOA = anterior preoptic area, mPOA = magnocellular preoptic area, SC = the suprachiasmatic nucleus, Str = striatum, TP = posterior tuberculum, VH = ventral hypothalamus, VP = ventral pallium.

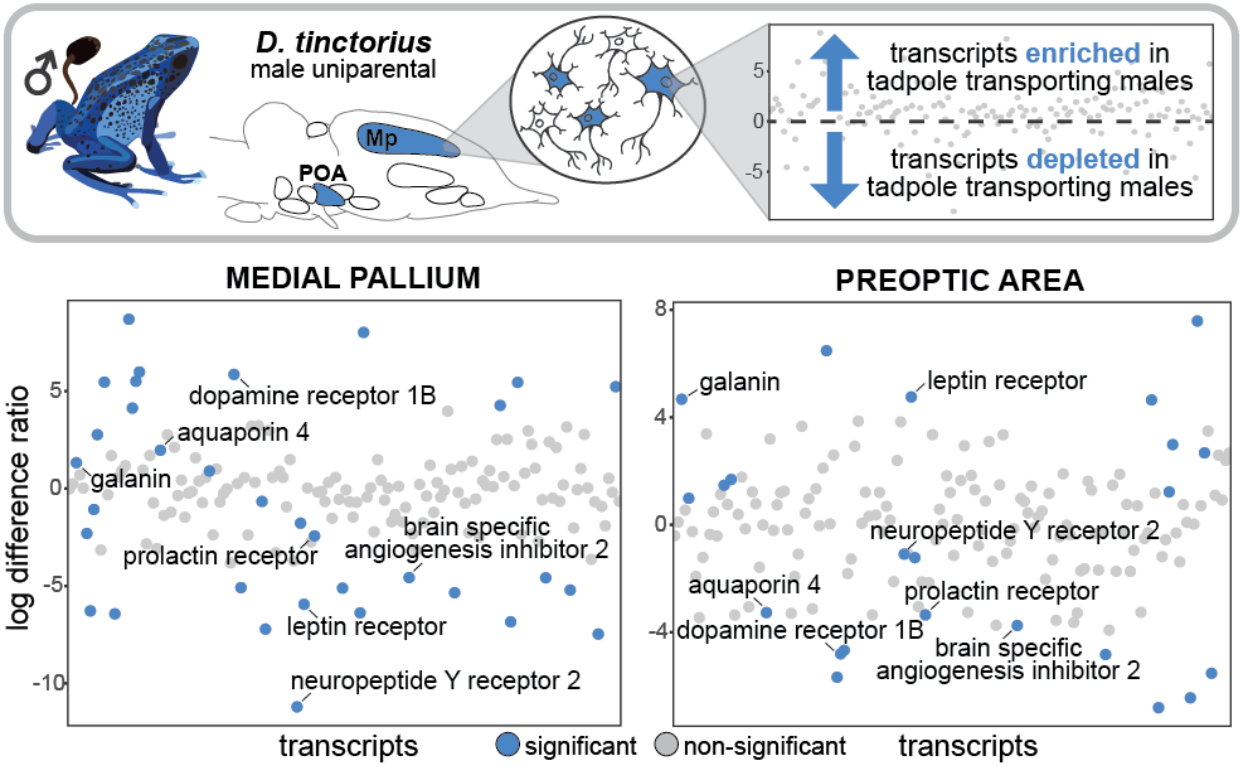


Figure 2. Gene expression in behaviorally relevant neurons. We identified significant expression differences in neurons active during parental care in the preoptic area (POA) and medial pallium (Mp) of tadpole transporting versus non-parental *D. tinctorius* males. We found some unique and some shared transcripts differentially expressed across brain regions (i.e. distribution of blue dots between plots). Those transcripts with significant expression enrichment or expression depletion in tadpole transporting males as compared to control males are highlighted in blue, and the seven transcripts overlapping between brain regions are labeled. The same candidate transcripts are plotted in the same order along the x-axis for both brain regions.

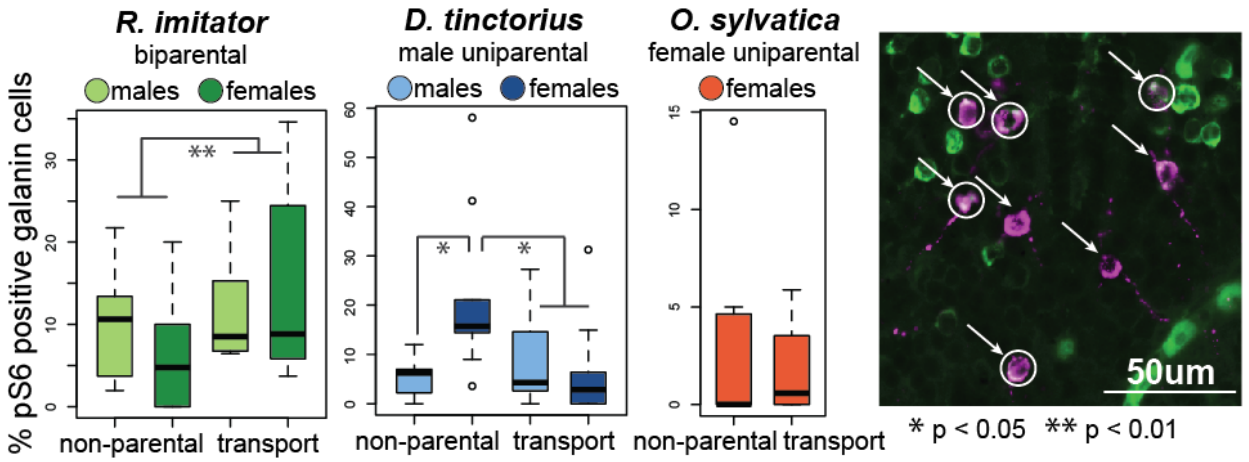


Figure 3. Preoptic area galanin neuron activity. Parental *R. imitator* had a greater proportion of active galanin neurons, as did the female partners of non-parental *D. tinctorius*. Representative micrograph: magenta = galanin positive neurons (arrows), green = pS6 positive neurons, white = co-localization indicating active galanin neurons (circles).