

1 **The neural basis of tadpole transport in poison frogs**

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16 **Running Title:** Mechanisms of parental care in poison frogs

17 **Word count:** 5,286 (including references)

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

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27 **Abstract**

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

## 41 **Background**

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

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

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

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

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

82

83

## 84 **Methods**

### 85 *Laboratory sample collection*

86 *Dendrobates tinctorius* and *Ranitomeya imitator* frogs were housed in breeding pairs in  
87 the laboratory, allowing us to identify both parental individuals and their non-caregiving partners.  
88 To control for effects of experience, all pairs successfully reared at least one clutch from egg-  
89 laying through tadpole transport prior to the experiment. For the non-parental group, we collected  
90 frog pairs between parental bouts when they were not caring for eggs or tadpoles, collecting  
91 individuals of both the caregiving sex (non-transport; n=10 *D. tinctorius*, n=7 *R. imitator*) and their  
92 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



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

201 Our primary objective was to utilize phosphoTRAP data to identify Mp and POA cell types  
202 whose activity differed between tadpole transport and non-parental individuals. To this end, we  
203 restricted further analysis to a subset of 158 transcripts representing cell types with known roles  
204 in parental care (Table S1). We identified transcripts as significantly enriched/depleted based on  
205 a combination of log-fold enrichment thresholds (>4) and permutation testing (Supplemental  
206 Materials). Permutation testing and visualization were done using R Statistical Software (version  
207 3.5.0; the R Foundation for Statistical Computing).

208

209

## 210 **Results**

### 211 *Neural induction during tadpole transport*

212 We compared neural activity patterns in tadpole transporters and their non-transporting  
213 partners across three closely related poison frog species with distinct parental care strategies  
214 (Fig. 1A). Differences in neural activity depended on behavioral group, sex, and brain region (Fig.  
215 1B; Table 1) and associations between behavioral group and neural induction were brain region  
216 specific (Table 1; group\*region: *D. tinctorius*:  $F_{1,2515}=5.00$ ,  $p<0.0001$ ; *R. imitator*:  $F_{12,557}=6.85$ ,  
217  $p<0.0001$ ; *O. sylvatica*:  $F_{12,557}=5.53$ ,  $p<0.0001$ ). We found overall differences between the  
218 transporting and non-transporting sex in male uniparental *D. tinctorius* (sex\*group\*region:  
219  $F_{1,2515}=3.89$ ,  $p<0.0001$ ) but not biparental *R. imitator* (Table 1; Fig. 1B). Indeed, *post hoc* analyses  
220 of region-specific differences revealed greater similarity between sexes in biparental and  
221 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

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

385

386

### 387 **Acknowledgements**

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

392

### 393 **Funding**

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

398

### 399 **Data availability**

400 Cell counts, read counts from phosphoTRAP, R code for phosphoTRAP analysis, and the  
401 *D. tinctorius* brain atlas are available as Supplemental Materials associated with the manuscript.  
402 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**

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521 **Tables & Figures**

522

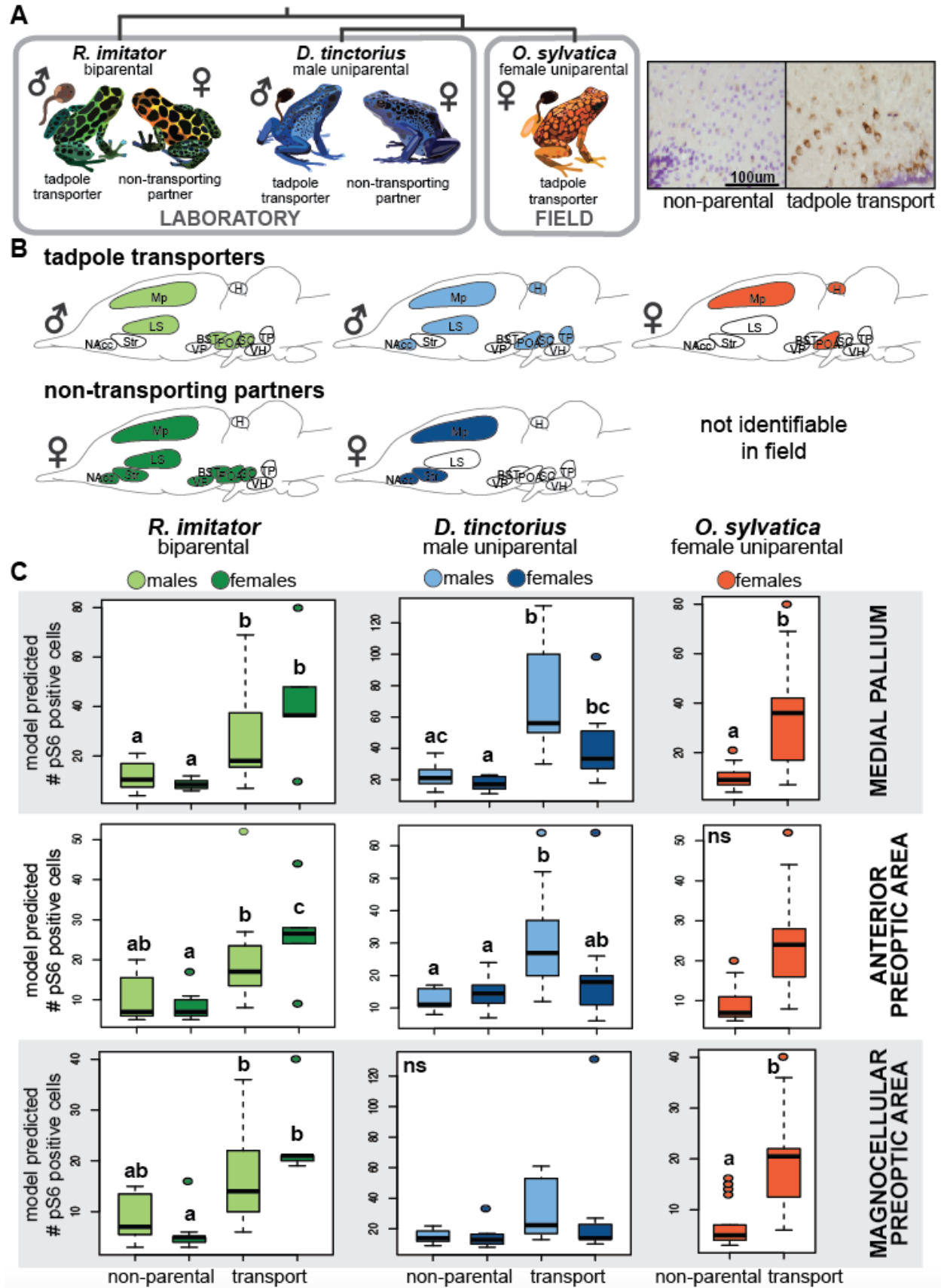
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**Table 1.** Summary of main statistical effects for neural induction differences.

		<b>df</b>	<b>F value</b>	<b>p value</b>
<b><i>R. imitator</i></b>	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
	n			
<b><i>D. tinctorius</i></b>	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
	n			
<b><i>O. sylvatica</i></b>	group	1,557	1.02	0.3126
	region	12,557	9.40	<0.0001
	group*region	12,557	5.53	<0.0001

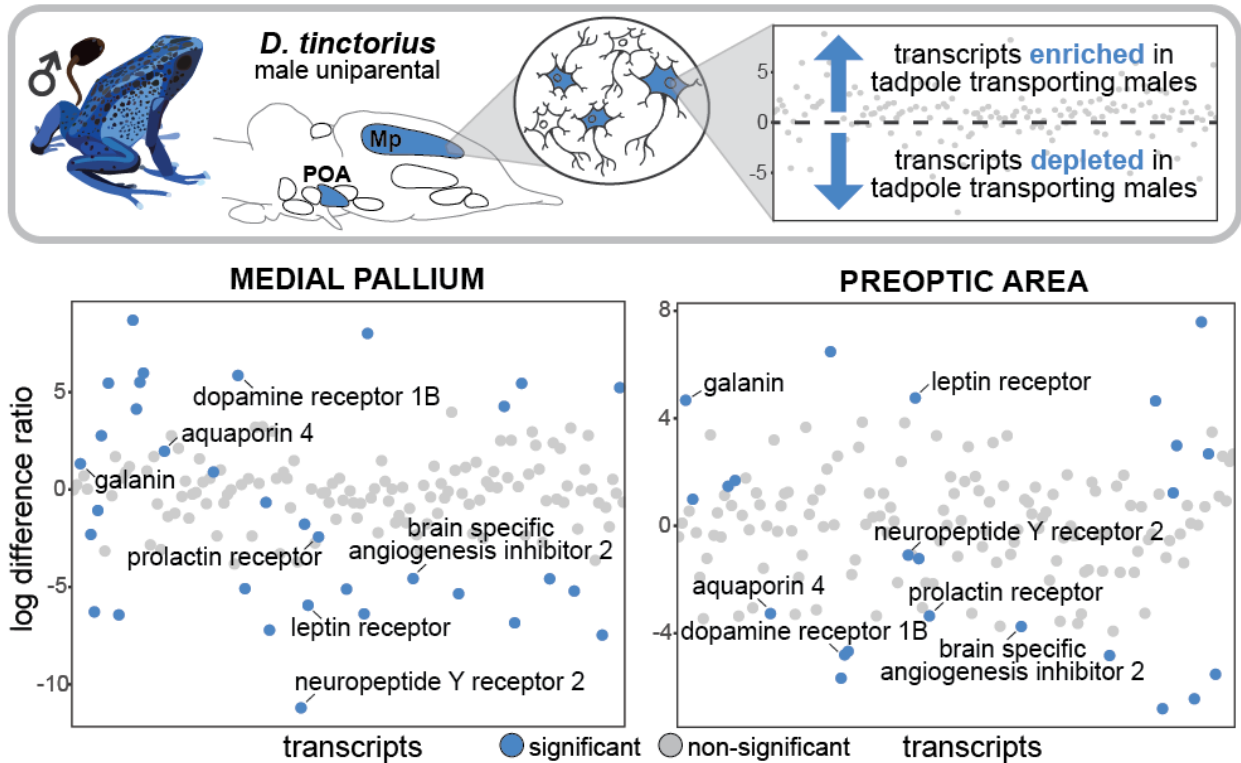
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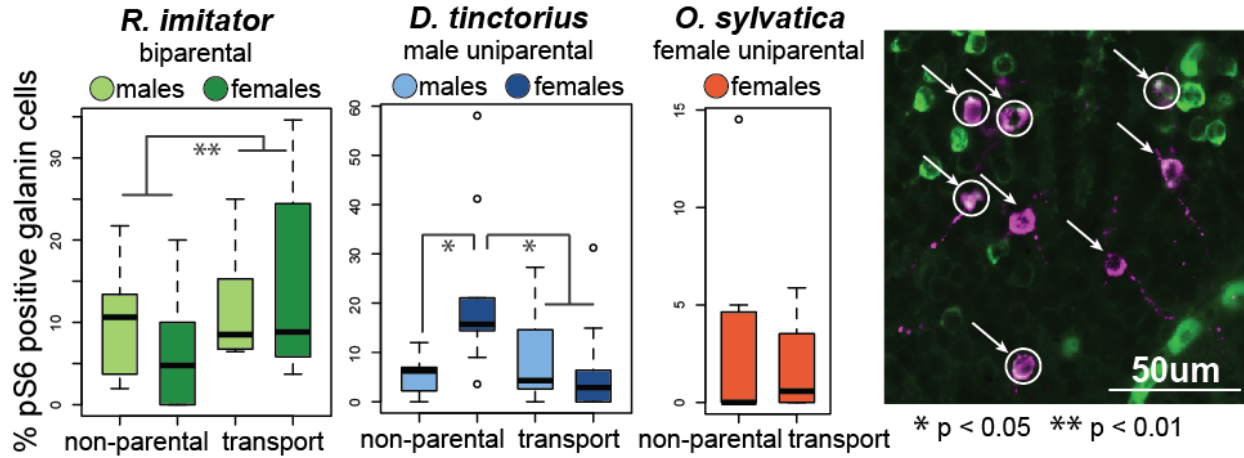


526 **Figure 1. Patterns of neural induction associated with parental care. (A)** Overview of  
527 experimental design. Our comparative approach allowed us to identify brain regions important in  
528 parental care independent of sex and species. **(B)** Overview of brain regions showing differences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

540



541  
542 **Figure 2. Gene expression in behaviorally relevant neurons.** We identified significant  
543 expression differences in neurons active during parental care in the preoptic area (POA) and  
544 medial pallium (Mp) of tadpole transporting versus non-parental *D. tinctorius* males. We found  
545 some unique and some shared transcripts differentially expressed across brain regions (i.e.  
546 distribution of blue dots between plots). Those transcripts with significant expression enrichment  
547 or expression depletion in tadpole transporting males as compared to control males are  
548 highlighted in blue, and the seven transcripts overlapping between brain regions are  
549 labeled. The same candidate transcripts are plotted in the same order along the x-axis for both brain regions.



550  
551

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