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

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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.

Background

41

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

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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.

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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

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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

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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

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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

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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).

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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).

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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

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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.

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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)

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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

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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

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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*

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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

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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.

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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.

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Authors' contributions

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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.

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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

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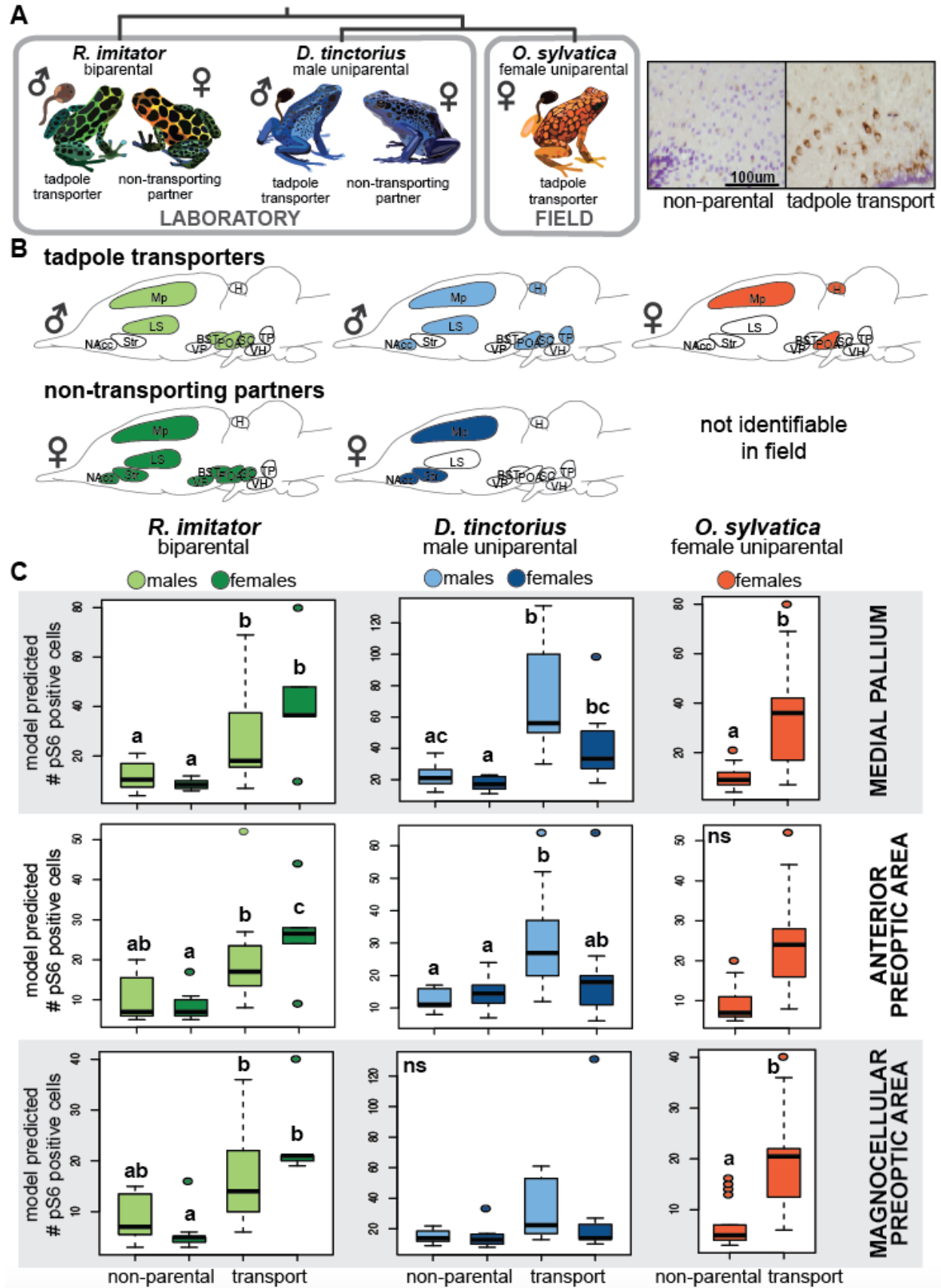
526 **Figure 1. Patterns of neural induction associated with parental care. (A)** Overview of
527 experimental design. Our comparative approach allowed us to identify brain regions important in
528 parental care independent of sex and species. **(B)** Overview of brain regions showing differences
529 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.

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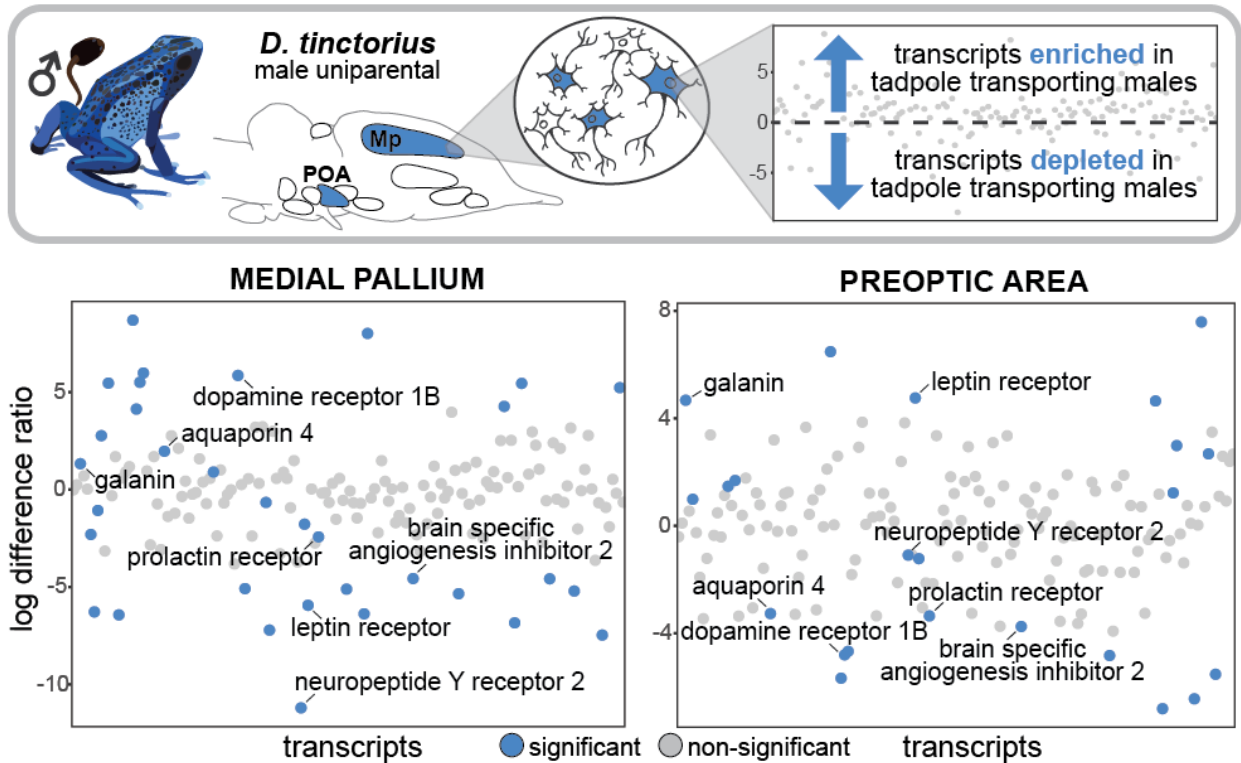
541
542 **Figure 2. Gene expression in behaviorally relevant neurons.** We identified significant
543 expression differences in neurons active during parental care in the preoptic area (POA) and
544 medial pallium (Mp) of tadpole transporting versus non-parental *D. tinctorius* males. We found
545 some 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 labeled. The
549 same candidate transcripts are plotted in the same order along the x-axis for both brain regions.
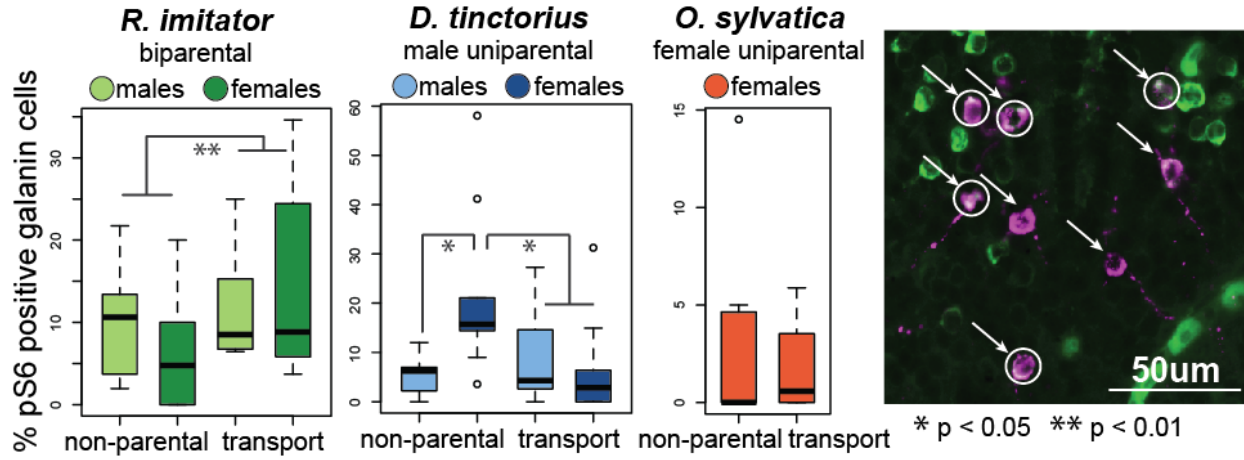
550
551
552 **Figure 3. Preoptic area galanin neuron activity.** Parental *R. imitator* had a greater proportion
553 of active galanin neurons, as did the female partners of non-parental *D. tinctorius*. Representative
554 micrograph: magenta = galanin positive neurons (arrows), green = pS6 positive neurons, white =
555 co-localization indicating active galanin neurons (circles).