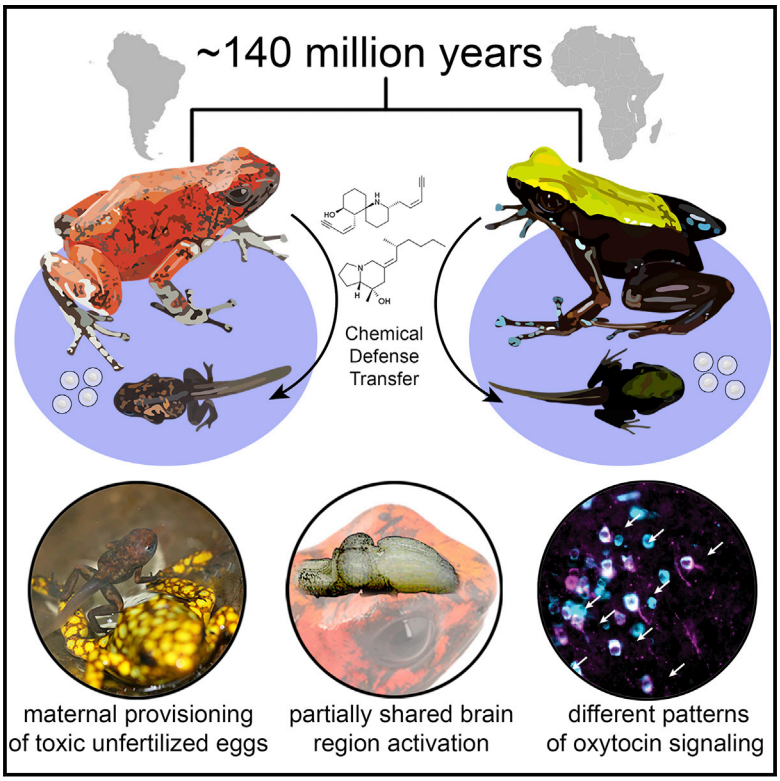
Report

Current Biology

Mechanisms of Convergent Egg Provisioning in Poison Frogs

Graphical Abstract



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In Brief

Maternal provisioning has evolved multiple times in amphibians, including in South American and Malagasy poison frogs. Field studies in Ecuador and Madagascar by Fischer, Roland et al. reveal that maternal care facilitates toxin provisioning and relies on similar brain regions in these clades with convergently evolved toxicity and maternal care.

Highlights

- South American and Malagasy poison frogs exhibit convergently evolved traits
- Both clades are toxic and provide parental care via maternal egg provisioning
- Egg-provisioning provides chemical defenses to developing tadpoles in both clades
- Provisioning relies on shared brain regions but distinct molecular mechanisms

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Mechanisms of Convergent Egg Provisioning in Poison Frogs

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SUMMARY

Parental provisioning of offspring with physiological products (nursing) occurs in many animals, yet little is known about the neuroendocrine basis of nursing in non-mammalian species. Within amphibians, maternal provisioning has evolved multiple times, with mothers of some species feeding unfertilized eggs to their developing offspring until tadpoles complete metamorphosis [1–3]. We conducted field studies in Ecuador and Madagascar to ask whether convergence at the behavioral level provides similar benefits to offspring and relies on shared neural mechanisms in dendrobatid and mantellid poison frogs. At an ecological level, we found that nursing allows poison frogs to provide chemical defenses to their tadpoles in both species. At the neural level, nursing was associated with increased activity in the lateral septum and preoptic area, demonstrating recruitment of shared brain regions in the convergent evolution of nursing within frogs and across vertebrates [4]. In contrast, only mantellids showed increased oxytocin neuron activity akin to that in nursing mammals [5], suggesting evolutionary versatility in molecular mechanisms. Our findings demonstrate that maternal provisioning provides similar potential benefits to offspring and relies on similar brain regions in poison frog species with convergently evolved toxicity and maternal care.

RESULTS

Convergence in Alkaloid Provisioning to Tadpoles

Studies in dendrobatids have established that egg provisioning is costly to mothers but benefits offspring [2, 6] and that tadpoles fed by their mothers carry alkaloids [7, 8]. Adult poison frogs acquire alkaloids from a diet of leaf-litter arthropods to which tadpoles do not have access. This suggests the transfer of chemical defenses from mothers to offspring could provide potential additional benefits beyond nutrition. To address this possibility, we tested whether convergently evolved nursing behavior in South American and Malagasy poison frog lineages provides tadpoles with chemical defenses [7]. We collected field samples from *Oophaga sylvatica* (the Little Devil poison frog) in Ecuador and *Mantella laevigata* (the Climbing Mantella) in Madagascar (Figure 1). We extracted and analyzed alkaloids from mothers' skin and oocytes, tadpoles' skin, eggs provisioned to tadpoles, and tadpole water pools. We detected 184 putative alkaloids across all *O. sylvatica* and 317 putative alkaloids across all *M. laevigata* samples (Data S1A and S1B). Toxin profiles were highly variable, and we therefore restricted analyses to 32 putative alkaloids shared by all nursing *O. sylvatica* mothers and 72 putative alkaloids shared by all nursing *M. laevigata* mothers to facilitate comparisons across individuals. Many of these alkaloids were also detected in tadpoles, supporting the hypothesis of toxin transfer from mothers to offspring (Figures 2A and 2B). Maternal alkaloids were also found in internal oocytes, trophic eggs laid to feed tadpoles, and in tadpole water pools (Figures 2A and 2B). Generally, the highest abundance of alkaloids was found in mothers and tadpoles, though patterns of relative abundance across tissues varied among alkaloids (e.g., Figure 2C).

Increased Neural Activity in the Preoptic Area and Lateral Septum of Nursing Females

We next tested the hypothesis that the convergent evolution of nursing behavior across South American and Malagasy poison frog lineages relies on shared neural mechanisms. To identify brain regions active during egg-provisioning, we compared patterns of neural activity between nursing mothers and non-nursing control females using an immunohistochemical marker for phosphorylated ribosomes (pS6) that serves as a general marker of neural activity [10]. We quantified neural activity in 13 candidate brain regions involved in parental and social behavior across vertebrates [11]. We found brain region-specific neural activity differences in nursing versus non-nursing females in both *O. sylvatica* (group*region: $F = 7.71$, $p < 0.0001$) and *M. laevigata* (group*region: $F = 3.46$, $p < 0.0001$). Two of the brain regions that showed nursing-specific increases in neural activity

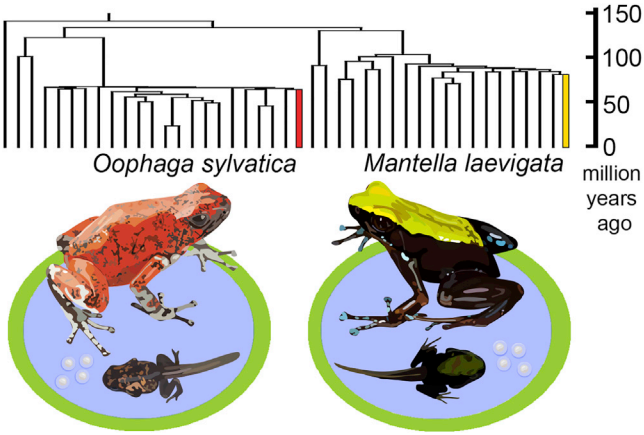

Figure 1. Nursing Behavior Has Evolved Independently in Amphibians

Malagasy mantellids and South American dendrobatids belong to different frog clades that diverged roughly 140 million years ago. While egg provisioning to developing tadpoles occurs across a variety of anuran families, *Oophaga sylvatica* (left) and *Mantella laevigata* (right) show convergence not only in maternal care but also in the evolution of aposematic coloration and alkaloid sequestration for chemical defense. Tree was pruned from [9]. Unlabeled branches represent other anuran families. For the fully labeled tree, see also Figure S3.

were shared between species: the medial preoptic area (*O. sylvatica*: F = 4.56, p = 0.033, *M. laevigata*: F = 5.63, p = 0.018) and the lateral septum (*O. sylvatica*: F = 18.77, p < 0.0001, *M. laevigata*: F = 3.84, p = 0.050) (Figure 3). We also identified several brain regions with species-specific activity patterns. Nursing *O. sylvatica* had increased activity in the medial pallium (F = 13.52, p = 0.0003) and decreased activity in the posterior tuberculum (F = 6.78, p = 0.009), while nursing *M. laevigata* had increased activity in the nucleus accumbens (F = 9.10, p = 0.003), the basolateral nucleus of the stria terminalis (F = 5.36, p = 0.021), the anterior preoptic area (F = 10.19, p = 0.0015), the ventromedial hypothalamus (F = 14.40, p = 0.0002), and the ventral pallium (F = 8.80, p = 0.0031) (Figure 3). Detailed statistical results are in Tables S1 and S2.

Opposite Patterns of Oxytocin Neuron Activity during Nursing Behavior in Mantellid versus Dendrobatid Poison Frogs

The preoptic area of the hypothalamus is critical for parental behavior across vertebrates [12], and the activation of preoptic area oxytocin neurons is important for nursing behavior in mammals [13, 14]. Behavioral and physiological differences between nursing in mammals and anurans notwithstanding, we examined a link between oxytocin signaling and nursing in poison frogs. We fluorescently co-labeled brain tissue for oxytocin and the pS6 marker of neural activity to quantify the percentage of oxytocin neurons active during nursing (Figure 4A). While the total number of oxytocin neurons did not differ between behavioral groups in either species (Figure S1), in *O. sylvatica* the proportion of active oxytocin neurons decreased during nursing (F = 18.77, p < 0.0001), while in *M. laevigata* the proportion of active oxytocin neurons increased during nursing (F = 15.55, p = 0.0015) (Figure 4B).

DISCUSSION

Nursing behavior is a specialized form of parental care that is particularly costly because it entails provisioning offspring with resources produced by the parents' own physiology. Nursing behavior in vertebrates includes lactation in mammals [15], crop milk feeding in birds [16], egg provisioning in amphibians [1], and skin-feeding in fish [17] and caecilians [18]. This remarkable behavior requires coordinated physiological and neural changes, many of which remain poorly understood. Although some shared brain regions (notably the preoptic area) and molecules (notably oxytocin and prolactin) have been implicated in vertebrate parental care generally (reviewed in [4]), studies on the neuroendocrine basis of offspring provisioning outside of mammals are virtually absent (but see [19] for an example in birds). A perspective from outside the mammalian lineage is needed to determine whether alternative mechanistic "solutions" can facilitate analogous provisioning behavior or whether the convergent evolution of nursing behavior has occurred via repeated recruitment of similar underlying mechanisms.

We explored provisioning of alkaloids and neuroendocrine mechanisms of nursing across South American and Malagasy poison frogs that diverged roughly 140 million years ago. While egg-provisioning has evolved in a number of anuran families [1], dendrobatids and mantellids have independently evolved alkaloid-based chemical defenses and warning coloration in addition to nursing [20–22]. We found that nursing females in both clades transfer alkaloids to their developing tadpoles and that egg-provisioning behavior relies on partially shared neural mechanisms. We suggest that the transfer of chemical defenses along with nutrients provides an additional benefit of this costly maternal behavior.

Mothers Provision Tadpoles with Chemical Defenses

Poison frogs do not synthesize the alkaloids they carry but rather sequester chemical defenses from their diet of leaf litter arthropods [23], to which aquatic tadpoles do not have access. Although the fertilized egg clutches of several amphibians contain toxins [24], these toxins dissipate during development and tadpoles tend not to carry chemical defenses [25]. Alkaloid transfer via egg provisioning has been demonstrated in another dendrobatid, *Oophaga pumilio* [7, 8] in which chemically defended tadpoles have a survival advantage [26]. We found that both *O. sylvatica* and *M. laevigata* provision their tadpoles with alkaloids, suggesting convergence in both nutritional and chemical defense provisioning in South American and Malagasy poison frogs.

The predominant mechanism of alkaloid transfer remains uncertain because alkaloids could be transferred via trophic eggs and/or released from the mother while she is in the water pool and subsequently absorbed by tadpoles. We cannot distinguish between these alternatives at present because we found alkaloids in both tadpole water pools and eggs; however, we have established that both oocytes and trophic eggs contain alkaloids, highlighting the likely transfer via egg provisioning. The presence of alkaloids in tadpole water pools is an interesting observation that may have implications for tadpole survivorship because tadpoles only develop granular glands for alkaloid storage during metamorphosis [27] and a toxic water pool may

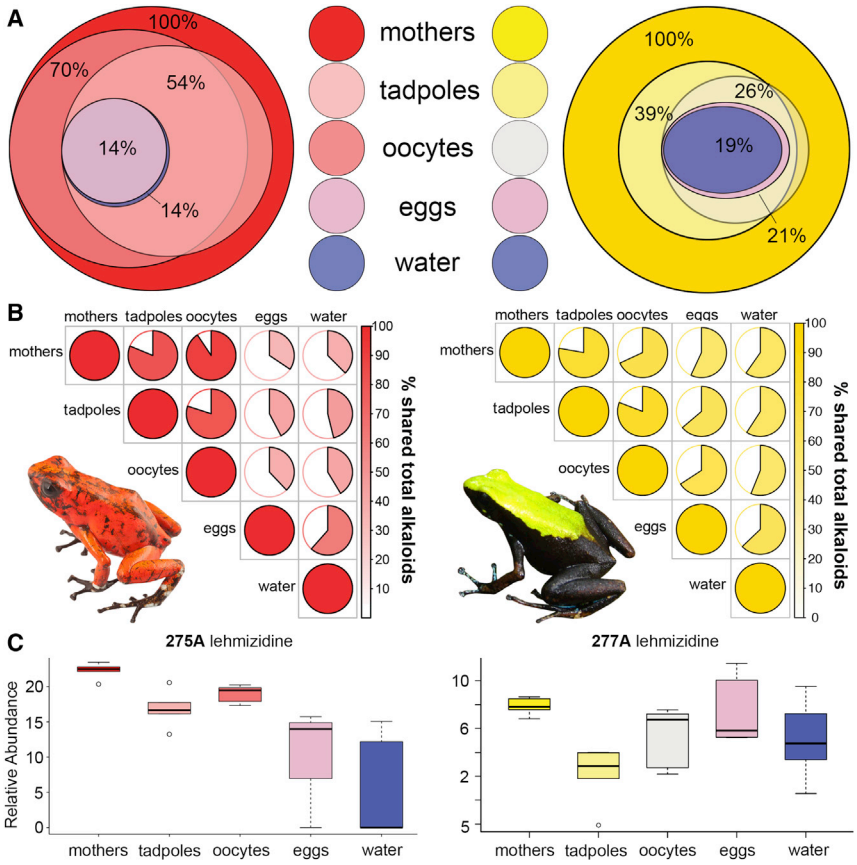

Figure 2. Mothers Provision Tadpoles with Alkaloids across Independent Lineages of Poison Frogs

(A) Percentage of overlap of maternal skin alkaloids with alkaloids in tadpole skin, internal oocytes, trophic eggs laid for tadpoles, and water from tadpole deposition sites in *O. sylvatica* (left) and *M. laevigata* (right). The collection of toxins shared across all adult females (32 in *O. sylvatica*, 72 in *M. laevigata*) is considered 100%. Average percentages were calculated from values for each tadpole and tissue paired with its mother.

(B) Matrices show the percentage of shared alkaloids across pairwise sample comparisons.

(C) Relative toxin abundances (measured on an arbitrary scale relative to nicotine) for representative lehmizidine alkaloids from *O. sylvatica* (left) and *M. laevigata* (right). Data are represented in box plots where boxes represent the first and third quartiles, the line within the box indicates the median, whiskers show the minimum and maximum values, and dots are outliers.

See also Data S1 and S4.

defend pre-metamorphic larvae against predators, competitors, and/or pathogens. By demonstrating alkaloid transfer to tadpoles and alkaloid presence in tadpole pools, we set the stage to dissect the mechanisms and fitness consequences of toxin transfer in future.

Shared Brain Regions Associated with Maternal Provisioning Behavior

We found recruitment of some shared brain regions in the convergent evolution of nursing behavior in South American and Malagasy poison frogs. In both species, nursing was associated with increased activity in the lateral septum and preoptic area. The lateral septum modulates goal-directed and social behavior [28–30]. This broad function encompasses a role in social recognition and memory [31–33], including in the context of parental care [34]. Although there is little functional information concerning the role of the lateral septum in amphibians, roles for goal-directed motivation and social recognition during nursing are apparent: mothers perform this costly behavior over the course of months, returning at regular intervals to provision offspring spread across multiple locations. Notably, offspring recognition in poison frogs appears to be based primarily on spatial cues [35, 36] providing an opportunity to explore how distinct sensory cues (e.g., spatial cues in frogs versus olfactory and auditory cues in mammals and birds) are integrated by shared neural circuits during parental behavior.

Preoptic area activity is associated with parental care across vertebrates, including in mammals [5], birds [37, 38], frogs [39], and fish [40]. Recruitment of the preoptic area in the independent evolution of parental care across vertebrates suggests this brain region represents a core node in parental care neural circuitry independent of sex, species, and care behavior specific phenomena. Indeed, we recently demonstrated sex- and species-independent increases in preoptic area activity during another parental care behavior (tadpole transport) in three species of dendrobatid poison frogs with distinct care strategies (male uniparental, female uniparental, and biparental) [39]. Our findings here expand on this previous observation by demonstrating increased preoptic area activity during a distinct parental behavior (nursing) and in a phylogenetically independent clade of frogs (Malagasy mantellids).

Non-shared Brain Regions Associated with Maternal Provisioning

In addition to increased neural activity in shared regions, we observed species-specific activity patterns in a number of brain regions. These differences may be experimental and/or biological in nature, and additional lab-based studies will be necessary to distinguish alternatives. From an experimental perspective, a major difference between our South American and Malagasy field sites was the existence of semi-naturalistic enclosures that allowed us to control social groups in Ecuador but not Madagascar. In addition, we collected control and nursing *M. laevigata* on separate trips in the dry and wet seasons, respectively. We note that both *O. sylvatica* and *M. laevigata* breed year-round, and reduced breeding in the dry season results primarily from a reduction in the number of suitable/stable tadpole deposition pools. While we cannot exclude long-term differences in neural circuit tuning and activity resulting from differences in climate and/or interactions with conspecifics, pS6 activity peaks ~45 min following behavior,

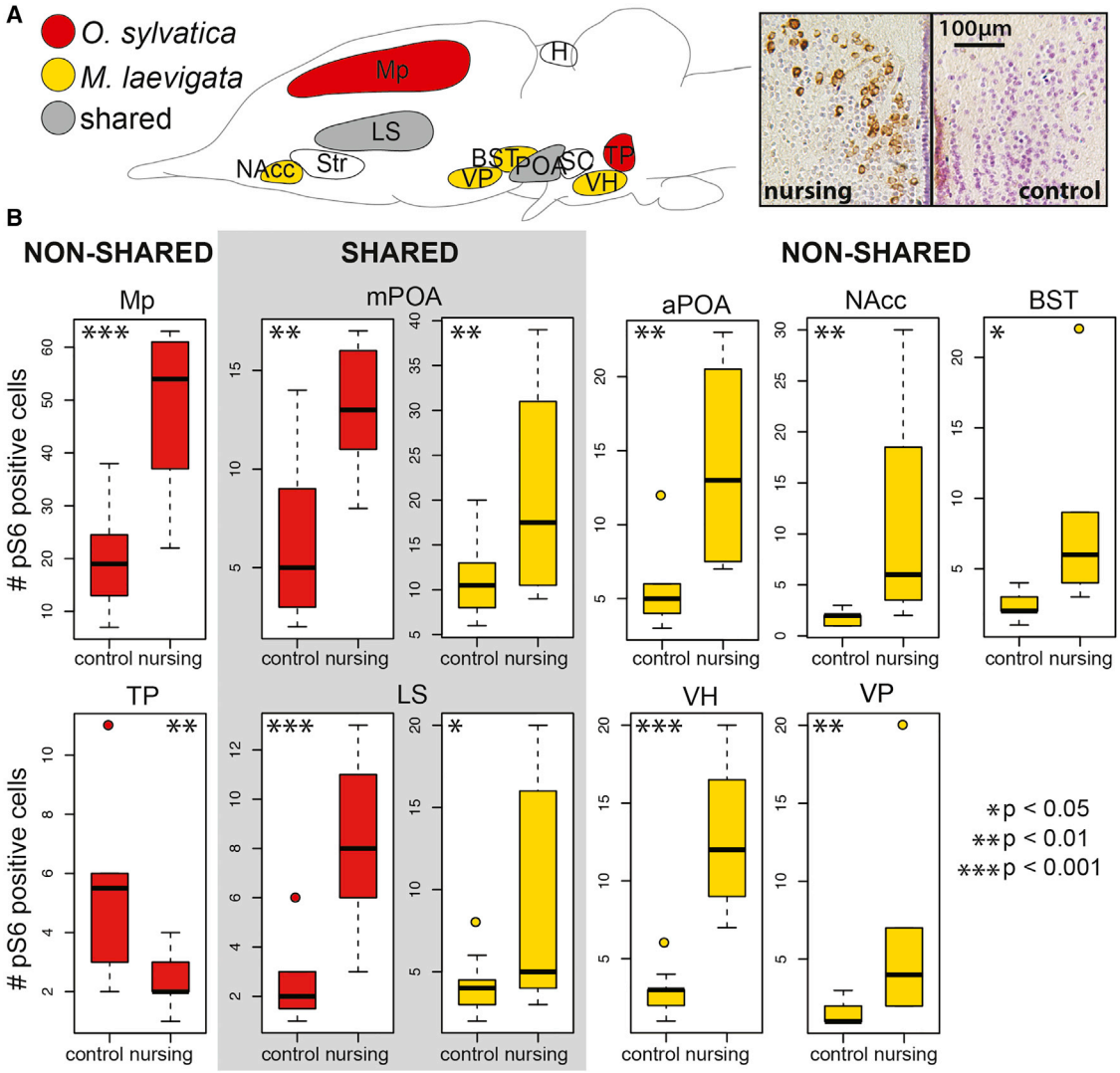

Figure 3. Neural Activity Differences Associated with Nursing Behavior in Independent Poison Frog Lineages

(A) Schematic showing brain regions with nursing-associated neural activity patterns in South American *O. sylvatica* (red), Malagasy *M. laevigata* (yellow), and shared across both poison frog clades (gray). Representative micrographs from the mPOA of *M. laevigata* are shown at right; scale bar, 100 microns.

(B) Detailed results for shared (center, gray box) and non-shared (left and right) brain regions exhibiting statistically significant activity differences in nursing versus control females (*p < 0.05, **p < 0.01, ***p < 0.001). Data are represented in box plots where boxes represent the first and third quartiles, the line within the box indicates the median, whiskers show the minimum and maximum values, and dots are outliers.

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, medial preoptic area; SC, the suprachiasmatic nucleus; Str, striatum; TP, posterior tuberculum; VH, ventral hypothalamus; VP, ventral pallium.

See also Tables S1 and S2 and Data S2 and S3.

and we can therefore exclude immediate effects of maternal or reproductive behavior in control females because we collected these females when they were alone and distant from tadpole deposition sites.

At a biological level, we note two key differences in the life history of *O. sylvatica* and *M. laevigata*. First, breeding sites and tadpole deposition sites are distinct in *O. sylvatica*, whereas tadpole deposition sites are also used for breeding in *M. laevigata*. Although we collected females of both species only when no other adult frogs were present, species-level differences in the association between nursing and breeding (i.e., interactions with males) could nonetheless lead to differences in neural activity associated with these behaviors. Second, tadpoles in *O. sylvatica* actively beg their mothers for meals by vibrating and nibbling along their legs and abdomen, while this begging behavior has not been observed in *M. laevigata*. Thus, neural activity differences during nursing could also be related to differences in interactions among mothers and offspring.

Finally, we note that most of the regions in which we observed species-specific activity patterns belong to the dopaminergic reward system. Homologies of the mesolimbic reward system

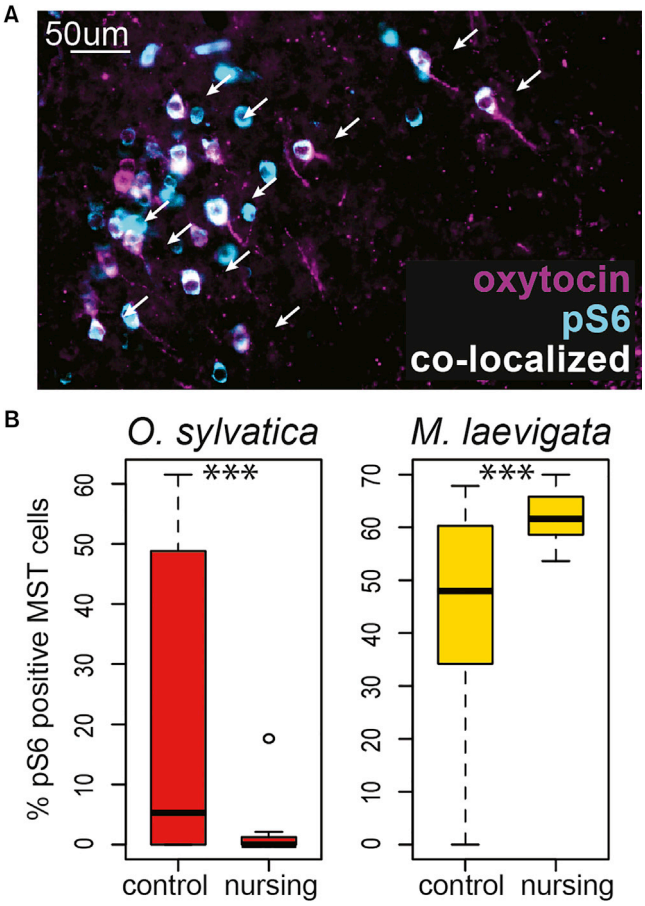

Figure 4. Opposite Patterns of Oxytocin Neuron Activity during Nursing in Dendrobatid and Mantellid Poison Frogs

(A) Representative image from the magnocellular preoptic area of *M. laevigata*. Oxytocin neurons are magenta, pS6 positive neurons are cyan, and active oxytocin neurons (i.e., those co-expressing oxytocin and pS6) are white (indicated by arrows); scale bar, 50 microns.

(B) The proportion of active oxytocin neurons is decreased during nursing in *O. sylvatica* and increased during nursing in *M. laevigata* (***$p < 0.002$). There was no difference in mean oxytocin neuron number (Figure S1). Data are represented in box plots where boxes represent the first and third quartiles, the line within the box indicates the median, whiskers show the minimum and maximum values, and dots are outliers.

See also Figures S1 and S2 and Data S2 and S3.

among vertebrates remain problematic, in particular, in amphibians and fish, in which data suggest homologs of the mammalian system may be spread across a number of brain regions [41]. Thus, the species-specific neural activity patterns we observe in *O. sylvatica* and *M. laevigata* may represent alternative circuit-level "solutions" for motivating goal-directed aspects of maternal care.

Oxytocin and Maternal Care

The idea that alternative mechanistic solutions may underlie convergent behavioral phenotypes is further supported by activity patterns of preoptic area oxytocin neurons during nursing. We observed increased oxytocin neuron activity during nursing in *M. laevigata* but decreased oxytocin neuron activity during nursing in *O. sylvatica*, demonstrating contrasting roles for oxytocin signaling during maternal care in poison frogs. Other studies exploring behavioral modulation by oxytocin in frogs give similarly contrasting results, finding increased aggression following oxytocin injections in *Eleutherodactylus coqui* [42] but no effect of oxytocin on parental behavior in the dendrobatid *Ranitomeya imitator* [43]. Taken together, these studies and our own suggest a nuanced role for oxytocin in amphibian social behavior, with variations linked to species-level differences in social behavior and life history.

Conclusions

Our work demonstrates that convergent nursing behavior in dendrobatid and mantellid poison frogs facilitates alkaloid provisioning in both clades and relies on partially shared brain regions but distinct cell types, suggesting that multiple mechanistic solutions may promote vertebrate maternal behavior. While additional work is necessary, we emphasize the value of our comparative design in identifying species-level differences and thereby facilitating hypothesis generation and testing with regards to the mechanisms underlying convergent behavioral adaptation across clades. The independent evolution of provisioning behavior across vertebrates provides an exciting opportunity to understand how similar evolutionary advantages of offspring provisioning lead to the targeting of shared versus distinct neural and physiological mechanisms.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.10.032.

A video abstract is available at https://doi.org/10.1016/j.cub.2019.10.032#mmc7.

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AUTHOR CONTRIBUTIONS

L.A.O. conceived of the study, obtained funding, directed the research, and performed brain immunohistochemistry; L.A.C. and M.V. contributed to study design and edited the grants; A.B.R., E.K.F., and N.R. collected samples in Madagascar; E.K.F., E.E.T., and N.A.M. collected samples in Ecuador; N.A.M. performed alkaloid extractions; C.V. and S.A.T. performed mass spectrometry; A.B.R. performed microscopy and cell counting; E.K.F. sectioned brains, performed cell counting, and analyzed cell-count data; C.V., L.A.O., N.A.M., and E.K.F. analyzed alkaloid data; and E.K.F. and L.A.O. wrote the paper with contributions from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Phospho-S6 Ribosomal Protein [Ser235/236]	Cell Signaling	Cat# 2211S; RRID: AB_331679
Oxytocin	Millipore Sigma	Cat# MAB5296; RRID: AB_11212999
Alexafluor 488 anti-rabbit	Life Technologies	Cat# A11034; RRID: AB_2576217
Alexafluor 594 anti-mouse	Life Technologies	Cat# A11029; RRID: AB_138404
Chemicals, Peptides, and Recombinant Proteins		
DL-Nicotine-(*methyl*-d_3)	Sigma-Aldrich	Cat#489077-50MG
HPLC grade Methanol	VWR International	Cat#10051183
Critical Commercial Assays		
ABC Kit	Vector Laboratories	Cat# PK-6100
3′,3′-diaminobenzadine (DAB) Kit	Vector Laboratories	Cat# SK-4100
Deposited Data		
LC-MS/MS raw data	DataDryad	10.5061/dryad.573n5tb3j
Experimental Models: Organisms/Strains		
Oophaga sylvatica	wild	N/A
Mantella laevigata	wild	N/A
Software and Algorithms		
SAS 9.4	https://www.sas.com	N/A
R	https://www.r-project.org/	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Dr. Lauren A. O'Connell (loconnel@stanford.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Frogs were collected from field sites in Ecuador and Madagascar. *Oophaga sylvatica* were collected at field sites near La Florida, Santo Domingo de los Tsáchilas Province, Ecuador in April and May of 2016 (N = 17). These field sites include naturalistic enclosures populated with *O. sylvatica* frogs that deposit tadpoles in artificial pools. We collected control females from enclosures containing only females to ensure these frogs were non-maternal at the time of capture. *Mantella laevigata* were collected on the island reserve of Nosy Mangabe, Madagascar in November 2015 (N = 11 controls) and July 2016 (N = 5 nursing). In Madagascar, frogs were free-roaming making it difficult to control for maternal state. To minimize the likelihood that control females were maternal, control females were captured when they were not in the vicinity of a pool and only in areas in which pools were devoid of tadpoles at the time of capture. In addition, we collected controls only in the dry season when there were limited pools for reproduction.

METHOD DETAILS

Tissue collection

The sampling protocol for both species was identical. After identifying a pool containing a tadpole, we waited until females arrived and entered the pool. We left females undisturbed until they exited the pool, upon which we captured them, and verified that they had provisioned the tadpole with eggs. We collected tissue from maternal females 30 min after they entered the pool containing a tadpole and only from those females that laid trophic eggs (N = 9 *O. sylvatica*; N = 5 *M. laevigata*). Control females were collected at matched times of day (N = 8 *O. sylvatica*; N = 11 *M. laevigata*). Frogs were anesthetized with application of 20% benzocaine gel to the belly and euthanized by rapid decapitation. Brains were removed and placed in 4% paraformaldehyde for 24 hours followed by storage in 1X phosphate buffered saline (1X PBS). The dorsal skin and oocytes were stored in 1 mL of methanol in glass vials for later alkaloid analysis. Other frog tissues were preserved either in 100% ethanol or RNAlater (Thermo Scientific, Waltham, MA, USA). In addition to adult tissues, we collected skin from tadpoles and trophic eggs that were stored in 1 mL methanol in glass vials. We also collected water from tadpole pools. Muscle and skeletal tissue were deposited in the amphibian collections of Centro Jambatu de

Investigación y Conservación de Anfibios in Quito, Ecuador (*O. sylvatica*) and in the Zoology and Animal Biodiversity Department of the University of Antananarivo, Madagascar (*M. laevigata*).

All samples were collected and exported in accordance with Ecuadorian (Collection permits: 005-15-IC-FAU-DNB/MA and 007-2016-IC-FAU-DNB/MA; CITES export permit 16EC000007/VS issued by the Ministerio de Ambiente de Ecuador) and Malagasy (Collection permits: 242/15/MEEMF/SG/DGF/DSAP/SCB and 140/16/MEEMF/SG/DGF/DSAP/SCB; CITES export permit: 1051C-EA12/MG15 and 679C-EA08/MG16 issued by the Direction Générale des Forêts et Direction des Aires Protégées Terrestres (Forestry Branch and Terrestrial Protected Areas Directorate of Madagascar)) laws. The Institutional Animal Care and Use Committee of Harvard University approved all procedures (Protocol 15-03-239).

Detection of alkaloids by mass spectrometry

Alkaloids were extracted as described in detail elsewhere [44] and briefly summarized below. Prior to extraction, skin samples were weighed with an analytical scale. Trophic eggs and oocytes were processed in a similar manner as skins, except that starting material was not weighed. The entire contents of each sample vial (all tissue and the methanol in which it was stored) were emptied into a sterilized Dounce homogenizer. To ensure the transfer of all materials, the empty vial was rinsed with 1 mL of methanol, which was also added to the homogenizer. We added 25 μg of D3-nicotine (Sigma-Aldrich, St Louis, MO, USA) in methanol to each sample to serve as a standard. Samples were ground with the piston ten times in the homogenizer before being transferred to a glass vial. The homogenizer was rinsed with an additional 1 mL of methanol in order to collect all residual alkaloids, and this methanol was also added to the final glass vial. Samples were stored at −20°C until further processing. Alkaloids from water samples were extracted using Oasis HLB VAC RC 30 mg extraction cartridges (Waters Corporation, Milford, Massachusetts, USA) on a vacuum manifold according to manufacturer instructions, including washes of 5% methanol and elution with 100% methanol. To avoid clogging the cartridges, debris were removed from water samples with a coarse sieve prior to processing.

Alkaloids were analyzed using liquid chromatography / tandem mass spectrometry (LC-MS/MS). Samples were run on a Thermo Q-Exactive Plus and a Phenomenex Gemini C18 3 μm 2.1 × 100 mm column (Torrance, CA, USA). Mobile phase A was composed of water with 0.1% formic acid, and mobile phase B was composed of acetonitrile with 0.1% formic acid. The flow rate was 0.2 ml/ min. The gradient began with 0% B for one min, then increased linearly to 100% B at 15 min, and held until 18 min. The column was then re-equilibrated to initial conditions for 3 min before the next sample. Blanks were run at regular intervals to ensure no carry over.

Alkaloids were tentatively identified by comparing this LC-MS/MS dataset to a dataset obtained by gas chromatography / mass spectrometry (GC/MS) from the same samples and used in a previous study exploring environmental variation and chemical defenses in the same frogs [45, 46]. The alkaloids detected by GC/MS in the previous study were identified using mass spectral data provided in Daly et al. [47]. For the LC-MS/MS data in the present study, a Tracefinder (Tracefinder 4.0, ThermoFisher Scientific) library was created with the accurate mass of all frog alkaloids from the Daly database and used to identify and integrate all potential poison frog alkaloids in the samples. This allowed more sensitive detection of alkaloids in all samples compared to GC/MS and permitted us to trace which potential alkaloids were present in the various sample types. Correlation with the previous dataset for the same frogs allows us to select the mostly likely poison frog alkaloid when several candidates with the same mass were present. Files from LC-MS/MS are available on DataDryad (https://doi.org/10.5061/dryad.573n5tb3j).

Brain immunohistochemistry

Brains were transferred from 1X PBS to 30% sucrose solution for cryoprotection at 4° C for 48 hours, embedded in mounting media (Tissue-Tek® O.C.T. Compound, Electron Microscopy Sciences, Hatfield, PA, USA), and rapidly frozen on dry ice. Brain samples were stored at −80° C until cryosectioning into four coronal series at 14μm. After sectioning, slides were dried for 1–3 hours and stored at −80° C until further processing.

We performed immunohistochemistry to assess levels of neural activity as well as the overlap between active neurons and cell types of interest. We used an antibody for phosphorylated ribosomes (Phospho-S6 Ribosomal Protein [Ser235/236] Antibody #2211, Cell Signaling, Danvers, MA, USA) as a proxy of neural activation [10]. We followed standard immunohistochemical procedures for 3′,3′-diaminobenzadine (DAB) antibody staining. Briefly, we quenched endogenous peroxidases in 30% sodium hydroxide solution, blocked slides in 5% normal goat serum, and incubated slides in primary antibody (pS6, 1:500 in a 2% normal goat serum solution with 0.3% Triton X-100) overnight at room temperature. The following day, slides were incubated in secondary antibody for 2 hours, followed by avidin-biotin complex (ABC Kit; Vector Labs, Burlingham, CA, USA) solution for 2 hours, and treatment with DAB (Vector Labs) for 2 min. We washed slides with 1X PBS before and between all of the above steps. Finally, slides were rinsed in water, counterstained in cresyl violet, dehydrated in a series of ethanol baths (50%, 75%, 95%, 100%, 100%), and cleared with xylenes prior to coverslipping with Permount Mounting Medium (Fisher Scientific).

We performed fluorescent co-labeling for pS6 and oxytocin on alternate sections of the same brains used for the pS6 staining described above. We followed the same general procedure, but incubated slides in a mix of primary antibodies for pS6 (1:500) and oxytocin (1:5000, MAB5296; Millipore Sigma, Burlington, MA, USA) and a mix of fluorescent secondary antibodies (1:200; Alexafluor 488 (pS6) and 594 (oxytocin)). Following incubation in secondary antibody solution, slides were rinsed in water and immediately coverslipped with Vectashield with DAPI (Vector Labs). Antibody specificity was confirmed by lack of signal after pre-incubating the oxytocin antibody with the mesotocin peptide (Bachem, Torrance, CA, USA), which blocked all signal (Figure S2).

Microscopy and cell counting

Stained brain sections were photographed at 20x magnification. Brightfield sections were imaged on a Zeiss AxioZoom microscope (Zeiss, Oberkochen, Germany) connected to an ORCA-ER camera (Hamamatsu, San Jose, CA, USA). Fluorescent sections were imaged on a Leica DM4B compound light microscope connected to a fluorescent light source. We quantified labeled cells from photographs using FIJI image analysis software [48]. Cell number was counted in a single hemisphere for each brain region in each section in which that region was visible. For pS6, we measured the area of candidate brain regions and counted all labeled cells within a given region. We quantified cell number in thirteen brain regions that modulate social decision-making across vertebrates [11]: the nucleus accumbens (NAcc), the basolateral nucleus of the stria terminalis (BST), the habenula (H), the lateral septum (LS), the magnocellular preoptic area (Mgv), the medial pallium (Mp; homolog of the mammalian hippocampus), the anterior preoptic area (aPOA), the medial preoptic area (mPOA), the suprachiasmatic nucleus (SC), the striatum (Str), the posterior tuberculum (TP; homolog of the mammalian ventral tegmental area), the ventral hypothalamus (VH), and the ventral pallium (VP). For the pS6 co-stain with oxytocin, we quantified cells only in the preoptic area (both aPOA and mPOA), as this is where oxytocin cell bodies are concentrated in the amphibian brain. We counted oxytocin-positive cells, pS6 positive cells, and the number of pS6-oxytocin co-labeled cells.

QUANTIFICATION AND STATISTICAL ANALYSIS

Neural activity differences

We analyzed differences in neural activation associated with egg provisioning behavior using generalized linear mixed models. Behavioral group (nursing versus control), brain region, and their interaction were included as main effects predicting the number of pS6 positive cells using a negative binomial distribution appropriate for count data with unequal variances. Individual was included as a random effect to control for the fact that multiple sections of the same brain region and multiple brain regions were quantified for each frog. Rather than averaging cell counts across brain regions, we included frog identity as a repeated-measure to control for both random and systematic variation across the large number of tissue sections quantified for each individual. Brain region area was included as an offset variable to control for body size differences between frogs, known size differences between brain regions, and rostral to caudal size/shape variation within brain regions. We explored main effects of group, sex, and regional differences in further detail using post hoc comparisons Tukey adjusted for multiple hypothesis testing. Due to differences in the timing of sample collection and processing, we analyzed data for the two species separately. Sample sizes were N = 8 control and N = 9 nursing *O. sylvatica*, and N = 11 control and N = 5 nursing *M. laevigata*. Results are in Figure 3 and Tables S1 and S2. Raw cell counts and statistical code are in the Data S2A, S2B, and S3.

Oxytocin cell number and activity

We tested for differences in the number and activity of oxytocin neurons using generalized linear mixed models. To compare the number of neurons, we included behavioral group as a main effect predicting the number of oxytocin neurons using a negative binomial distribution as before. To analyze activity differences in oxytocin neurons, behavioral group predicted the proportion of pS6 positive oxytocin neurons using a binomial distribution. All statistical analyses were performed separately for each species using SAS Statistical Software v. 9.4; (SAS Institute for Advanced Analytics) and plots were made using the base package in R v. 3.5.0 (The R Foundation for Statistical Computing). Raw cell counts and statistical code are in Data S2C, S2D, and S3.

Alkaloid analysis

We restricted comparisons of chemical profiles between nursing mothers, tadpoles, internal oocytes, trophic eggs, and tadpole water to those alkaloids present across all provisioning females within each species. For each tadpole, oocyte, and trophic egg sample we calculated the proportion of alkaloids in common with the mother's skin sample. We then averaged the percent overlap within each species; we are not able to directly compare across species as sample sets were run on different columns. We visualized average percent overlap across all samples using the plotrix v. 3.7-4 in R. We also calculated percent overlap in a pairwise fashion for all tissues. Pairwise overlap was calculated using the proportion of alkaloids shared between an individual tissue group pair to the total number of alkaloids across both tissues. We visualized matrices of pairwise comparisons using the corr.plot v. 0.84 in R (Data S4).

Finally, we also plotted variation in the level of single toxins. Toxin levels are measured as the integrated area of the major adduct extracted ion chromatogram divided by the area of the internal standard (D3 Nicotine). To make values comparable across species, we normalized these values based on the lowest measurable sample for each species. We plotted log abundance values to facilitate visualization across mothers, tadpoles, oocytes, trophic eggs, and water samples which differ in toxin abundance by orders of magnitude. We note that we retained log of zero values as zero because these are biologically meaningful despite being mathematically undefined. We refer to normalized, log-transformed values simply as 'relative toxin abundance' (Data S1C and S1D). Boxplots were generated using the base package in R v. 3.5.0 (The R Foundation for Statistical Computing).

DATA AND CODE AVAILABILITY

The raw and transformed toxin abundances (Data S1A–S2D), raw cell counts are in Data S2A–S2D, and representative analysis code (Data S3 and S4) are included as Supplemental Data associated with the manuscript. The accession number for the LC-MS/MS data reported in this paper is Dryad: https://doi.org/10.5061/dryad.573n5tb3j.