



Research

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Pouch brooding marsupial frogs transfer nutrients to developing embryos

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Marsupial frogs have a unique reproductive mode in which females carry eggs enclosed in a sealed dorsal brood pouch. While most anurans are considered to be oviparous with lecithotrophic eggs, the extensively vascularized membrane of the brood pouch in marsupial frogs suggests potential opportunities for nutrient transfer. We tested for matrotrophy in the live-bearing *Gastrotheca excubitor* (Hemiphractidae), through feeding insects labelled with a ^{13}C -fatty acid and a ^{15}N -amino acid to brooding marsupial frogs. We observed significant increases of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in both maternal pouch tissues and embryos, suggesting nutrient transfer. Embryo dry mass also increased with developmental stage, providing further direct evidence for matrotrophy. These results suggest that in addition to gas exchange, the vascularized brood pouch membrane of *G. excubitor* also enables maternal nutrient transfer. This finding revealed a suspected but untested trait in the evolution of parental care in marsupial frogs, in contrast to previous work on *Gastrotheca* species that release tadpoles, and suggests greater complexity in reproductive and provisioning modes than previously thought.

1. Introduction

Anurans exhibit a great diversity of reproductive modes [1], ranging from typical aquatic eggs with free-feeding tadpoles, to terrestrial eggs with direct-developing larvae, as well as live-bearing where embryos or larvae are carried internally within pouches or oviducts of parents [2,3]. A consequence of brooding embryos enclosed within the body is that parents must provide respiratory gas exchange, often via a close connection between highly vascularized parental surfaces and egg membranes or gill structures of larvae [2,4,5]. While this close connection can provide opportunities for maternal nutrient transfer (i.e. matrotrophy), the vast majority of frogs, including live-bearing species, are thought to be lecithotrophic, by which mothers provide all nutrients to larvae via yolk [2].

Across vertebrates, embryos or larvae can obtain nutrients by absorption through skin or gills, by ingesting maternal tissues from oviductal epithelia or mucosa, by cannibalizing eggs or siblings, or via placental structures [6,7]. Despite the strong potential for maternal nutrient transfer in egg brooding frogs, there have been few direct tests of matrotrophy beyond two early studies of an oviduct and a pouch brooding frog [2,4,8]. Direct evidence of matrotrophy includes shifts among eggs, embryos and hatchlings in chemical composition, increasing embryonic dry mass, or transfer of isotopically labelled molecules or nutrients [7,9–11].

In this study, we tested the hypothesis that matrotrophy occurs in live-bearing *Gastrotheca excubitor* (Hemiphractidae; figure 1), a marsupial frog species that gives birth to froglets from fully enclosed pouches. We directly tested for maternal nutrient transfer in *G. excubitor* through feeding insects enriched in carbon (^{13}C) and nitrogen (^{15}N) isotopes to brooding mothers, which is an effective method for tracking nutrient allocation [10,12]. We also tested for matrotrophy by assessing if dry mass increased from eggs through developing

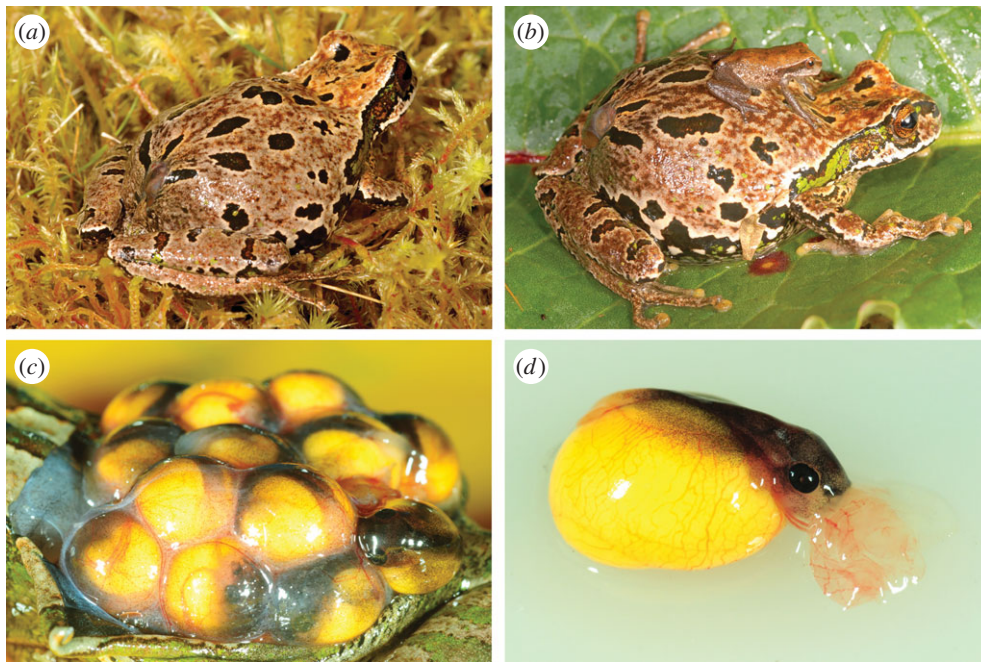


Figure 1. (a) Marsupial frogs (*Gastrotheca excubitor*) brood their direct-developing larvae within a fully enclosed pouch, from which (b) froglets emerge. (c) Embryos are surrounded by a highly vascularized membrane within the brood pouch, which along with (d) external gills enables gas exchange and nutrient transfer throughout development. Photographs by A. Catenazzi.

embryos. With these data coupled with a comparative exploration of Hemiphractidae, we tested the hypothesis that pouch structure and embryonic retention influence the evolution of matrotrophy among marsupial frogs.

2. Material and methods

(a) Study species and experimental design

We captured brooding *G. excubitor* frogs near Manu National Park, Peru [13] from 1 to 23 June 2014. We transported frogs to Wayqecha Biological Station within 4 h of capture, weighed them and transferred them to individual 1.2 l plastic containers with ventilated lids, wet paper towels and fresh leaves. We monitored the frogs daily, noting general condition and feeding. The containers were cleaned weekly and the frogs weighed. The frogs were maintained for 26–48 days until the end of the experiment on 19 July 2014.

We performed a feeding experiment among frogs randomly assigned to two groups. Frogs in the treatment ($n = 5$) group were fed one isotopically enriched prey per day, whereas frogs in the control group ($n = 3$) were fed the same amount of non-enriched prey (electronic supplementary material, table S1). We regularly sampled the feeder insects for isotope analysis. At the end of the feeding experiment, the frogs were euthanized (20% benzocaine gel applied to the pelvic patch) and dissected for embryos and maternal tissues. During dissections, all scissors and forceps were rinsed with ethanol and flame sterilized between removal of each tissue to prevent cross-contamination. After counting, measuring and staging the embryos [14], all tissues were rinsed with ethanol and then sealed in individual Whirl-Pak bags with 95% ethanol until preparation for isotope analysis.

(b) Stable isotope treatment and analysis

Treated frogs were fed insects coated with $\delta^{13}\text{C}$ -palmitic acid ($1\text{-}^{13}\text{C}$, 99%; Cambridge Isotope Labs, Inc.) dissolved in ethanol, and $\delta^{15}\text{N}$ -leucine (^{15}N , 98%) dissolved in water. Treated insects were isotopically enriched with $\delta^{15}\text{N}$ ($33.2 \pm 7\%$ Air (see below))

and $\delta^{13}\text{C}$ ($-6.8 \pm 5.1\%$ VPDB (see below)) compared with non-treated prey ($\delta^{15}\text{N}$: $4 \pm 0.9\%$; $\delta^{13}\text{C}$: $-25.3 \pm 0.8\%$) (log $\delta^{15}\text{N}$: $F_{1,23} = 22.2$, $p < 0.001$; $\delta^{13}\text{C}$: $F_{1,25} = 11.8$, $p = 0.002$; electronic supplementary material, table S1). Dissected tissues, embryos and whole insects were freeze dried and homogenized, and 0.25 mg powdered samples were placed into tin capsules for stable isotope analysis [12]. We report all isotope values in delta notation ($\delta X = [R_{\text{sample}}/R_{\text{standard}} - 1] \times 1000$) in parts per thousand relative to the international standards (Vienna Pee Dee Belemnite (VPDB) for $\delta^{13}\text{C}$ and Air for $\delta^{15}\text{N}$). Measurements were conducted on an elemental analyser and continuous flow isotope ratio mass spectrometer at Southern Illinois University Carbondale. The analytical precision of these analyses was $\pm 0.08\%$ for $\delta^{13}\text{C}$ and $\pm 0.08\%$ $\delta^{15}\text{N}$.

(c) Statistical analysis

To test for the effect of isotope labelling on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, we used linear models for insects and frogs, and mixed models for embryos. Both models included treatment as a fixed effect, and mixed models included embryo developmental stage as a covariate (frog identity was used as a random factor to account for non-independence of unfertilized eggs and embryos from the same female). Similarly, a mixed model was used to test for an association between embryo dry mass and developmental stage with frog identity as a random factor. To test whether developmental stage was associated with $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ in the embryos, we excluded control animals, and fitted linear and quadratic functions to data from treated animals. An F -test was then used to compare the fit of these linear and quadratic functions. $\delta^{13}\text{C}$ data were squared and $\delta^{15}\text{N}$ values were log-transformed to meet assumptions of normality. Data for this study are available via Dryad (doi:10.5061/dryad.g89s5) [15].

3. Results and discussion

We found that live-bearing females of *G. excubitor* provide maternally derived nutrients to their embryos, as demonstrated by two lines of evidence. First, embryos exhibited higher

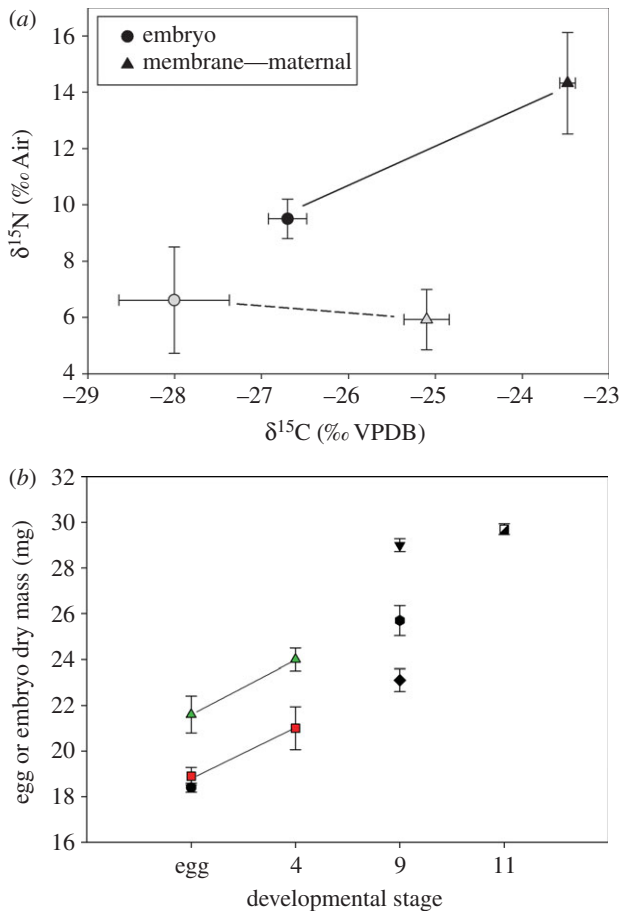


Figure 2. (a) Mean (\pm s.e.) $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ stable isotope values for the maternal membrane (triangles) and embryos (circles) of control (grey symbols) and labelled (black symbols) brooding marsupial frogs fed insects for six weeks; lines connect treatments. (b) Mean dry mass of embryos (\pm s.e.) increased with developmental stage. Each type of symbol represents embryos from an individual female ($n = 7$); each line connects unfertilized eggs and embryos from the same female. (Online version in colour.)

$\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in parallel with isotope enrichment of maternal pouch membranes and tissues in mothers fed isotopically labelled prey, when compared with control frogs and embryos. Maternal pouch membranes surrounding developing embryos were significantly enriched in the treated frogs (figure 2a) compared with control frogs for both $\delta^{15}\text{N}$ ($F_{1,5} = 9.1$, $p = 0.04$) and $\delta^{13}\text{C}$ ($F_{1,5} = 61.1$, $p = 0.001$). Embryonic $\delta^{15}\text{N}$ values were associated with maternal isotope labelling ($F_{1,7} = 7.0$, $p = 0.02$), along with developmental stage ($F_{1,5.1} = 7.7$, $p = 0.04$). Embryo $\delta^{13}\text{C}$ patterns only exhibited a trend with isotope treatment ($F_{1,5.1} = 5.5$, $p = 0.07$). The second line of evidence supporting matrotrophy in *G. excubitor* was increased embryo dry mass with developmental stage (figure 2b; slope = 0.76 ± 0.05), ranging from eggs to near-metamorphic larvae ($F_{1,1.2} = 203.9$, $p = 0.03$).

The differences in embryonic $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ enrichment combined with mass increases across ontogeny suggest that *G. excubitor* embryos absorb specific macronutrients to build additional body mass. Specifically, embryonic $\delta^{15}\text{N}$ increased with developmental stage in a nonlinear fashion among embryos of treated frogs (electronic supplementary material, figure S1a; $r^2 = 0.78$, d.f. = 5, $p = 0.02$), best described by a quadratic function ($p = 0.04$). By contrast, $\delta^{13}\text{C}$ was not associated with developmental stage (electronic

supplementary material, figure S1b; $p > 0.56$). Greater absorption of nitrogen relative to carbon nutrients parallels higher transfer of paternally derived amino acids in pouch brooding pipefish [10] and vocal sac brooding *Rhinoderma darwinii* [11]. In addition, the nonlinear $\delta^{15}\text{N}$ transfer to embryonic *G. excubitor* suggests amino acid demand increases with development in rapidly growing embryos and then declines near metamorphosis [16–18].

Among amphibians, known forms of parental nutrient transfer beyond egg yolk include uterine epithelial nutrient secretions and larval consumption of trophic eggs or siblings [2,19,20]. In our study, *G. excubitor* embryos apparently used their large, fused external gills to absorb dissolved nutrients that crossed egg membranes from the pouch lumen. Matrotrophy has been suspected in marsupial frogs owing to the highly vascularized external gills and pouch membrane [2,21], but previous studies found no evidence of embryonic mass change during development in *Gastrotheca riobambae* [4]. Thus, our study is the first, we believe, to directly demonstrate maternal nutrient transfer in a marsupial frog.

Brood pouch structure and the degree of embryonic retention probably determine matrotrophy. Pouch structure in hemiphraetid frogs ranges from simple dorsal skin patches to partially and fully enclosed pouches [5]. Frogs with fully enclosed pouches could be predisposed to transfer nutrients, because they must provide gas exchange for embryos. However, the degree of embryo retention may be a secondary determinant for matrotrophy because, while *G. riobambae* has a fully enclosed pouch, their embryos are released as free-feeding tadpoles [5]. By contrast, live-bearing frogs such as *G. excubitor* that give birth to froglets may be predisposed to matrotrophy, because of maternal and embryonic modifications necessary for sustained brooding. This hypothesis that pouch structure and embryonic retention influence the occurrence of matrotrophy in marsupial frogs is supported by comparison with other vertebrates [8,10].

Marsupial frogs are of great interest to developmental and evolutionary biologists because of the potential reversal in reproductive modes from live-bearing to tadpole development [22]. Our findings show that ancestral features for gas exchange for developing embryos may be co-opted via convergent evolution for nutrient transfer [7], and suggest that greater exploration of the diverse reproductive modes among marsupial frogs could provide insight into the evolution of matrotrophy in vertebrates.

Ethics. All institutional and national guidelines for the care and humane use of animals were followed and approved by the SIU IACUC (no. 14-009).

Data accessibility. Data for this study are available via Dryad (<http://dx.doi.org/10.5061/dryad.g89s5>) [15].

Authors' contributions. R.W.W. and A.C. conceived and co-designed the study, carried out the laboratory work, carried out the statistical analyses and drafted the manuscript. Both authors gave final approval for publication, and ensure the accuracy and integrity of the work.

Competing interests. The authors have no competing interests.

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