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HUSBANDRY REPORTS

Carotenoid Supplementation Enhances Reproductive Success in Captive Strawberry Poison Frogs (*Oophaga Pumilio*)

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Amphibians are currently experiencing the most severe declines in biodiversity of any vertebrate, and their requirements for successful reproduction are poorly understood. Here, we show that supplementing the diet of prey items (fruit flies) with carotenoids has strong positive effects on the reproduction of captive strawberry poison frogs (*Oophaga pumilio*), substantially increasing the number of metamorphs produced by pairs. This improved reproduction most likely arose via increases in the quality of both the fertilized eggs from which tadpoles develop and trophic eggs that are fed to tadpoles by mothers. Frogs in this colony had previously been diagnosed with a Vitamin A deficiency, and this supplementation may have resolved this issue. These results support growing evidence of the importance of carotenoids in vertebrate reproduction and highlight the nuanced ways in which nutrition constrains captive populations. Zoo Biol. XX:XX–XX, 2013.

Keywords: amphibian reproduction; carotenoids; captive breeding; *Dendrobates pumilio*; Dendrobatidae

STATEMENT OF THE PROBLEM

Amphibians are currently undergoing the most severe declines in species richness of any vertebrate [Hoffmann et al., 2010; Stuart et al., 2004]. Because amphibians are extremely abundant and play diverse trophic roles in both aquatic and terrestrial food webs [e.g., Davic and Welsh, 2004], these declines have troubling implications for both ecosystem health and the maintenance of diversity in other groups [McGrady-Steed et al., 1997; Naeem et al., 1994]. Given that threats to amphibian diversity are predicted to continue [Kriger and Hero, 2009], the establishment of ex situ breeding populations will be critical to conservation efforts, facilitating basic research and providing a source for re-introduction [Gascon et al., 2007]. Such captive breeding programs offer both opportunities and challenges because of a general lack of information about amphibian husbandry [e.g., Lee et al., 2006].

We investigated the effects of carotenoids on reproduction in a captive colony of strawberry poison frogs (*Oophaga pumilio*). This small, colorful poison frog, endemic to Central America, is threatened by habitat loss, climate change, and over-collection for the pet trade, and is protected under CITES Appendix II [Solis et al., 2010]. The species is also rapidly becoming a model species for evolutionary ecologists (e.g., special issue of *Ecology and Evolution* 27(4), July 2013), where the answers to numerous questions of interest, including how genetics control coloration and what proximate factors underlie variation in toxicity, rest on breeding experiments. However, previous attempts at captive breeding have been met with limited success, primarily due to high mortality during development [Summers et al., 2004]. Similarly, in the breeding colony we established (details below) mortality was initially high in larval and recently metamorphosed individuals.

A typical component of captive breeding is a dramatic simplification in animal diet. In the wild, prey consumption results from complex interactions between ecology (e.g., food availability and competition), physiology (e.g., nutritional requirements) and evolutionary history (e.g.,

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morphological and behavioral components of prey capture) [Toft, 1985]. In contrast, captive diet is necessarily limited to prey items that can be cultured effectively [e.g., Finke, 2013; Lee et al., 2006; Ogilvy et al., 2011], which may account for the frequent nutritional deficiencies in captive animals [Finke, 2013; Li et al., 2009; Ogilvy et al., 2012]. Successful breeding can be further complicated by changes in adult nutritional requirements associated with entering a reproductive state or requirements unique to early developmental stages [Brooks et al., 1997].

Carotenoids are a nutrient class particularly important during reproduction. These pigments confer the familiar yellow-orange color of egg yolk in diverse taxa [Blount et al., 2000], and while the mechanisms by which they contribute to animal health remains controversial [Møller et al., 2000], their ubiquitous inclusion in yolk along with evidence that they improve neonate health underline their importance for successful breeding [Blount et al., 2000; McGraw et al., 2005]. These pigments may also be important because they can be converted to Vitamin A by animals [de Pee and West, 1996; Slifka et al., 1999]. Vitamin A deficiency is common in captive frogs [Sim et al., 2010], and was indicated in this study by necropsies of juvenile *O. pumilio* that completed metamorphosis in captivity in Panama (performed by A. Pessier, Amphibian Disease Laboratory, San Diego Zoo). During a pilot study of the role of carotenoids in *O. pumilio* coloration, we noticed an increase in reproductive success. We report here on a detailed analysis of these patterns.

DESCRIPTION OF THE PROCESS

In August 2009, we established a colony of *O. pumilio* at Tulane University (Louisiana) with wild-caught individuals from four populations in the Bocas del Toro archipelago, Panama (Tranquilo Bay, Isla Bastimentos: 9°15′8.03″N, 82° 8′43.30″W; Cemetery, Isla Bastimentos: 9°20′48.48″N, 82° 12′23.04″W; Isla Popa: 9°8′25.98″N, 82°7′39.11″W; Aguacate Peninsula: 9°12′47.13″N, 82°12′49.29″W). Breeding pairs were housed in plastic enclosures maintained in an environmental chamber at 22–27°C and ~60% relative humidity under a 12L/12D light cycle (similar to conditions in Bocas del Toro). Sheet moss and plants served as a substrate for egg deposition, and water-filled PVC tubes in each corner of the tank provided tadpole rearing sites. We fed pairs with ~50 adult *Drosophila melanogaster* and/or *D. hydei* three times weekly.

Initially, fruit flies were reared on media similar to most commercial preparations, but in February, 2011, we began augmenting media with carotenoid supplements (Table 1) as part of a pilot study of coloration. In final cultures, powdered red phaffia yeast (*Xanthophyllomyces dendrorhous*, formerly *Phaffia rhodozyma*) provided ~63 μg/g astaxanthin, powdered marine algae (*Dunaliella salina*) provided ~69–132 μg/g beta-carotene and ~8–15 μg/g of other carotenoids including alpha-carotene, lutein, zeaxanthin and cryptoxanthin, and Spirulina (*Arthrospira platensis*) provided ~19 μg/g of unspecified carotenoids; the carotenoid-supplemented media, thus, contained ~159–229 μg/g total carotenoids more than the unsupplemented media. On both diets, we dusted flies with a powdered supplement (Repashy Calcium Plus ICB, Repashy Ventures Inc., San Marcos, CA) containing 1,000 μg/g total carotenoids and 137.6 μg/g vitamin A. Every 3 months, all individuals were also treated with a topical vitamin A supplement (Aquasol A®) according to manufacturer's instructions.

Although sample sizes were small, we suspected that carotenoid supplementation of fruit fly media was increasing the rate at which tadpoles successfully completed metamorphosis, and so on August 15, 2011, began providing all pairs with flies reared on carotenoid-supplemented media. To examine the effects of this supplementation, we tracked the reproduction of 63 unique pairs (formed with 50 male and 52 female *O. pumilio*) from August 2009 to November 2012 (spanning both diet treatments); 18 of these pairs were intact during under both diet treatments. We estimated two metrics of reproductive success: (i) output of clutches, tadpoles and metamorphs, and (ii) transition success, estimated as the

TABLE 1. Composition of fruit fly growth media

Ingredient	Quantity (g)	Source
1. Dehydrated potatoes	16.7	Idaho Pacific Colorado Corp., Center, CO
2. Brewer's yeast	3.7	Lewis Labs, Westport, CT
3. Confectioner's sugar	4.9	Bakers and Chefs™, Bentonville, AR
4. Methylparaben (preservative)	2.7	Josh's Frogs, Owasso, MI
5. Baker's yeast	0.3	Red Star®, Lasaffre Yeast Corp., Milwaukee, WI
6. Tap water	80	
7. Spirulina	1.4	Brine Shrimp Direct, Ogden, UT
8. Marine algae (powdered)	0.7	Brine Shrimp Direct, Ogden, UT
9. Red phaffia yeast (powdered)	0.7	Brine Shrimp Direct, Ogden, UT

For both unsupplemented and carotenoid-supplemented diets, ingredients 1–6 were homogenized and allowed to solidify before the introduction of ~50 adult flies. For carotenoid-supplemented treatment only, ingredients 7–9 were suspended in water before homogenization. Cultures were incubated at 25°C until new flies emerged (~15 days). Prior to feeding, we dusted flies with a standard amphibian vitamin supplement (Repashy Calcium Plus ICB).

proportion of clutches that produced tadpoles and the proportion of tadpoles that became metamorphs.

To estimate the effect of diet on reproductive output, we used generalized linear mixed models (GLMMs) in which we entered either clutches, tadpoles, or metamorphs as the dependent variable, diet as a fixed factor, the number of days paired as a covariate, and pair identity, male identity nested within pair and female identity nested within pair as random factors; to account for the large number of observed zeros, we fit models using a negative binomial distribution. To assess the effect of diet on transition success, we fit GLMMs where clutches/tadpole or metamorphs/tadpole were entered as the dependent variable using the events/trial syntax, diet was entered as a fixed effect, and random effects were included as above. As a supplementary and more conservative analysis, we also compared these measures of reproductive success in the pairs that were reproductively active under both diets; these data were compared using paired-*t* tests when difference scores were normally distributed and sign tests when not. We used SAS version 9.2 for all analyses.

DEMONSTRATION OF EFFICACY

Pairs produced fewer clutches on the supplemented diet ($F_{1,86} = 19.2$; $P < 0.001$), but produced more tadpoles ($F_{1,66.4} = 4.7$; $P = 0.033$) and more metamorphs ($F_{1,62.8} = 37.9$; $P < 0.001$; Fig. 1). All three measures of reproductive output were positively associated with the number of days pairs were together (clutches: $F_{1,86} = 103.8$; $P < 0.001$; tadpoles: $F_{1,87} = 63.6$; $P < 0.001$; metamorphs: $F_{1,87} = 5.6$; $P = 0.020$). On the supplemented diet, more clutches transitioned to tadpoles ($F_{1,75} = 19.2$; $P < 0.001$) and more tadpoles transitioned to metamorphs ($F_{1,75} = 35.9$; $P < 0.001$; Fig. 2). The random effects did not explain a significant proportion of the variance in any metric of reproductive success (all $\chi^2 < 1.8$, df = 1, all $P > 0.298$).

Similarly, pairs that experienced both diets produced fewer clutches per day on the carotenoid-supplemented diet (paired $t_{16} = 2.7$; $P = 0.017$), but produced more tadpoles (paired $t_{17} = -2.64$; $P = 0.017$) and more metamorphs (paired $t_{17} = -4.18$, $P = 0.001$). For these pairs, clutches produced more tadpoles on the carotenoid-supplemented (mean ± SD, 1.14 ± 1.25) than un-supplemented (0.14 ± 0.25) diet (paired $t_{14} = -3.0$; $P = 0.010$). However, there was no difference in the proportion of tadpoles that became metamorphs in this small sample ($N = 5$; sign test: $P = 1.000$).

Carotenoid supplementation of their fruit fly prey increased the number of offspring that captive *O. pumilio* reared to independence, an effect most parsimoniously explained by an increase in the quality of eggs (fertilized and trophic) produced by females [Blount et al., 2000; McGraw et al., 2005]. Carotenoid-supplementation increased the number of tadpoles produced per clutch, leading to an overall increase in tadpole production. This effect, coupled with an increase in the rate at which tadpoles completed metamorphosis, led to the increase in metamorph production. Although tadpole and metamorph output was higher, the number of clutches produced by females was lower under carotenoid-supplementation. This decreased clutch production suggests that (i) oogenesis is not limited by carotenoid availability [see also Ogilvy et al., 2012], and (ii) reproductive (vs. tropic) egg production is suppressed when females are caring for tadpoles, as they were more often under the carotenoid-supplemented treatment.

The natural diet of *O. pumilio* consists primarily of ants and mites [Toft, 1995], with the latter the more carotenoid-rich food source [Olson, 2006]. As a replacement for the natural diet, carotenoid-supplemented fruit flies were

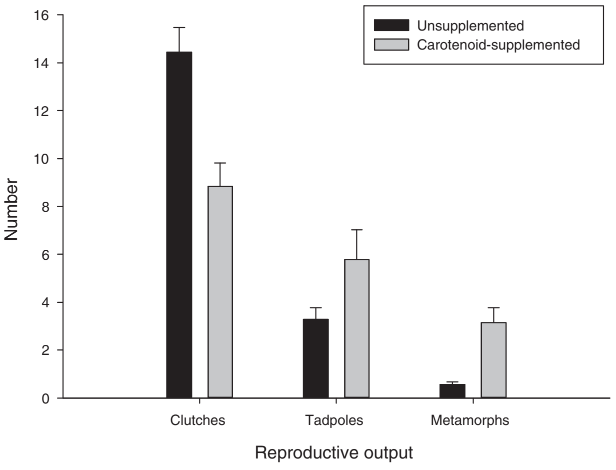

Fig. 1. Least square means (±SE) of the number of clutches, tapdoles and metamorphs produced by *O. pumilio* pairs fed an unsupplemented and carotenoid-supplemented fruit fly diet. Least square means are from a GLMM that included number of days paired as a covariate. The effect of diet was significant ($P < 0.001$) in all models.

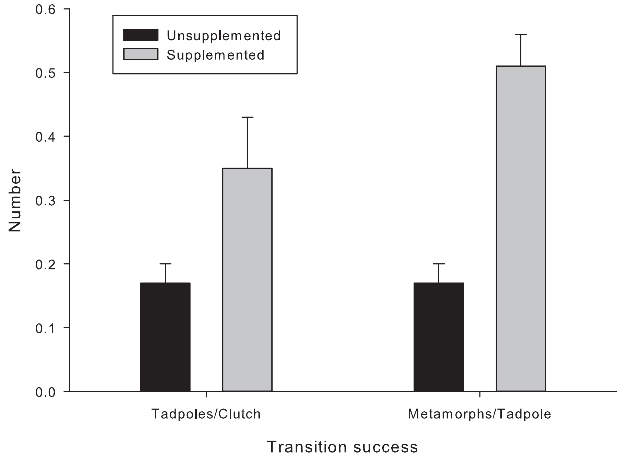

Fig. 2. Least square means (±SE) of the number of tapdoles produced per clutch and the number of metamorphs produced per tadpole by captive *O. pumilio* pairs fed an unsupplemented and carotenoid-supplemented fruit fly diet. The effect of diet was significant ($P < 0.001$) in all models.

superior to unsupplemented ones in our captive colony. Perhaps further highlighting the importance of prey item diet breadth, we found that wild insects attracted to fruit (primarily *Drosophila* spp.) allowed for successful reproduction by captive *O. pumilio* held outdoors at Bocas del Toro (*unpublished data*). Whether carotenoid-supplementation addressed a vitamin A deficiency in this ex situ population and whether that deficiency limited reproductive success remain unclear. Carotenoids are converted to vitamin A by other animals [de Pee and West, 1996; Slifka et al., 1999], but these metabolic pathways are poorly understood in amphibians. Because the conversion of various dietary carotenoids to vitamin A may be species-specific, supplements that provide a diverse suite of carotenoids may be of use when a species's metabolic pathways are unknown or when multiple species are reared with the same feeder cultures.

The effects of carotenoids on *O. pumilio* reproduction were far more dramatic than those reported for another colorful frog [*Agalychnis callidryas* Ogilvy et al., 2012], a pattern that likely arises because *O. pumilio* (and not *A. callidryas*) feed offspring with trophic eggs throughout development. Both obligate and facultative egg feeding occur in several anuran families [reviewed by Perry and Roitberg, 2006], and this intimate connection between parent and offspring nutrition can further complicate captive breeding; adults may appear healthy because their nutritional needs are met, but be unable to sufficiently provision offspring. Diet-induced reductions in egg quality should be considered as a candidate in any case of depressed developmental success in captive anurans. More broadly, both captive rearing and anthropogenic habitat alteration can shape and/or restrict the diet of animals [Isaksson and Andersson, 2007], and our results reveal how these constraints can cascade through the food web and between generations.

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