Effects of the Domesticated Cricket (*Acheta domesticus*) maintained with Broad Spectrum Carotenoids on Tiger Leg Tree Frog (*Phyllomedus tomopterna*) Coloration

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Introduction:

There is still much to be studied when it comes to captive amphibian nutrition. Recently an abundance of literature has shown specific nutrients that improve the overall health and reproductive success of amphibians in a captive setting. One type of nutrient, carotenes, is growing in popularity as they seem to have a positive effect on captive amphibians. Historically, calcium deficiency has caused issues with developing reptiles and amphibians; however plenty of literature has documented ways to prevent calcium deficiencies in a captive environment. A more common deficiency in captive amphibians is with Vitamin-A. Vitamin-A deficiencies can lead to several disorders including liver disease, bloating and short tongue disorder. As such, amphibian research has focused on ways to prevent Vitamin-A deficiencies in captive amphibians. Several studies have shown improvement in Vitamin-A levels using a variety of nutrients fed directly to amphibian prey. Spirulina, fish oil and carotenes have all been shown to improve growth, reproductive success and coloration in various species.

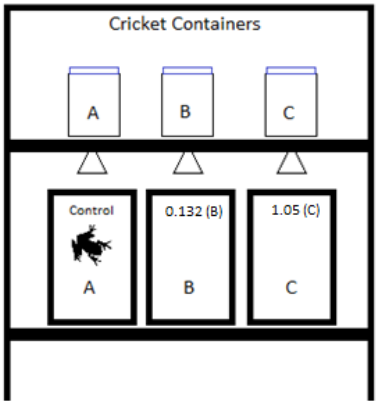
This study attempted to replicate previous studies using carotenoids as a feeder supplementation (Repashy SuperPig). We hoped to show, at the very least, broad spectrum carotenes fed to the Domesticated Cricket (*Acheta domestica*), will improve the coloration of Tiger Leg Tree Frogs (*Phyllomedusa tomopterna*) when ingested as it does for Red Eye Tree Frogs (*Agalychnis*

callidryas)(Ogilvy 2012). There are also implications to increased overall welfare, reproductive success and facility best practices with a high carotenoid feeder diets.

Methods:

Two clutches of Tiger Leg Tree Frog tadpoles were raised in an aquarium and separated at metamorphosis into three identical husbandry tanks (**Figure A**. Tank A, B and C). At metamorphosis, animals were separated via a "round robin" method to ensure average age of each tank was similar. The tanks were fed, serviced and cleaned in the exact same manner throughout the duration of the study. Each tank was furnished with similar tank furniture.

Figure A

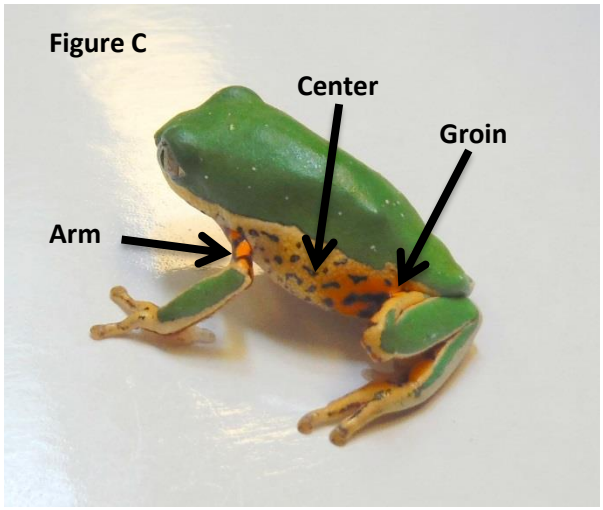


Crickets were housed in Kritter Karrier style enclosures kept above each husbandry (**Figure A**) tank with specifically formulated cricket diets and cricket quencher offered ad libitum. When Tiger Leg Tree frogs were fed, crickets were removed from each respective tank's Kritter Karrier, dusted with calcium and Vitamin D, and scatter fed to the animals. Three different cricket diets were used throughout the duration of the study (**Figure B**). A "Control" group (Tank A) was fed crickets maintained on a standard cricket maintenance diet (Timberline Cricket Power Food) devoid of any broad spectrum carotenoids. A "Trace" group (Tank B), were fed crickets maintained with trace concentrations of broad spectrum carotenoid (0.132 mg/g cricket diet). A "Test" group (Tank C) was fed a diet with substantially higher broad spectrum carotenoid concentrations (1.05mg/g cricket diet).

Figure B

Diet	Cricket Diet	SuperPig	Carotenoid Concentration (mg/g)
A	50g	0g	0
B	49g	1g	0.1322
C	42g	8g	1.058

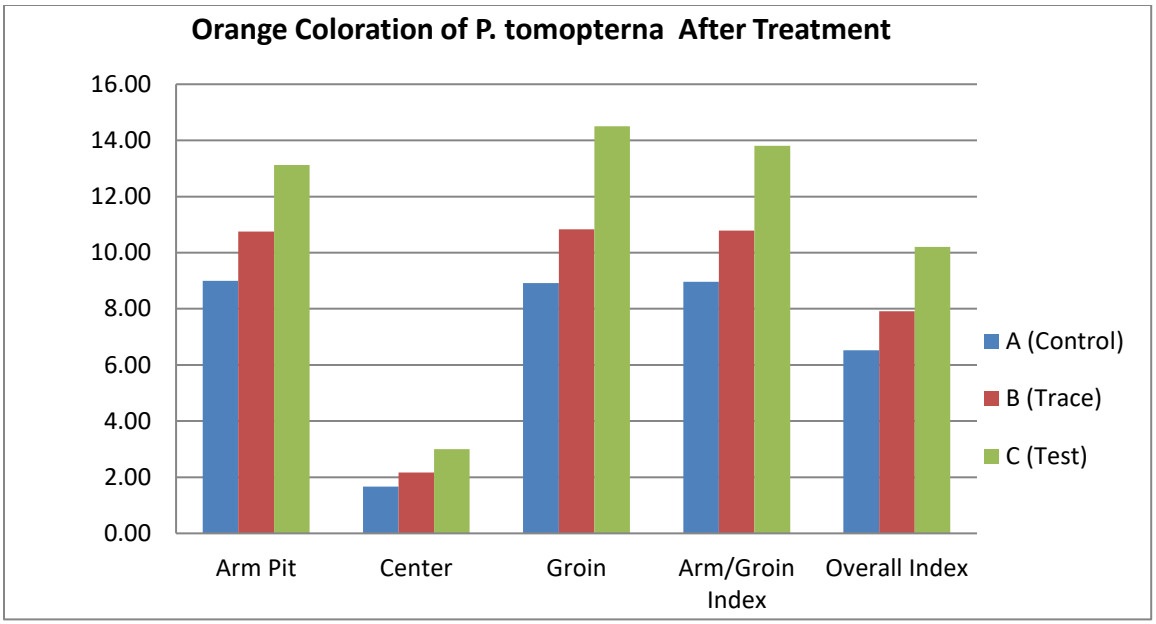
Tiger Leg Tree Frogs were maintained on supplemented crickets for approximately 3 months after the final metamorphosis. At the end of the study, an impartial researcher measured mass and orange coloration of the arm, center body and groin of each individual frog (Figure C). Orange coloration was quantified visually using a DSM Egg Yolk color wheel, who's values (1-15) are defined by CIE Standard colorimetric system. The measurements were combined using a non-weighted average to create an "Overall Index" (arm, groin and center) and "Arm/Groin Index" (Arm and Groin only). Indexes between groups were then compared using ANOVA for significance.



Results:

The results of Overall Orange Index Average for each group are shown in Figure D. The control group (Tank A), Trace (Tank B) and Test Group (Tank C) were found to have an index value of 6.53, 7.92, and 10.21 respectively.

Tank	Concentration (mg/g)	Weight (g)	Arm Pit	Center	Groin	Arm/Groin Index	Overall Index
A	**0**	1.80	9.00	1.67	8.92	8.96	**6.53**
B	**0.132**	1.12	10.75	2.17	10.83	10.79	**7.92**
C	**1.05**	1.77	13.13	3.00	14.50	13.81	**10.21**



Overall Index Single Factor ANOVA

SUMMARY				
Groups	***Count***	***Sum***	***Average***	***Variance***
Column 1	6	39.167	6.528	1.082
Column 2	6	47.500	7.917	0.753
Column 3	4	40.833	10.208	0.322

ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	32.53762	2	16.26881	20.85497	**8.77407E-05**	3.805565
Within Groups	10.1412	13	0.780093			
Total	42.67882	15				

Arm/Groin Index Single Factor ANOVA

SUMMARY				
Groups	***Count***	***Sum***	***Average***	***Variance***
Column 1	6	53.75	8.958	1.385
Column 2	6	64.75	10.792	2.360
Column 3	4	55.25	13.813	0.391

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	56.59505	2	28.29753	18.48485	**0.000158138**	3.805565
Within Groups	19.90104	13	1.530849			
Total	76.49609	15				

Center Body Single Factor ANOVA

SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	6	10	1.667	0.667
Column 2	6	13	2.167	0.567
Column 3	4	12	3.000	0.667

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.270833	2	2.135417	3.399235	**0.064942093**	3.805565
Within Groups	8.166667	13	0.628205			
Total	12.4375	15				

The Indexes created, arm and groin coloration were significantly different ($P<0.05$) between the three groups. Although slightly more orange between test groups, orange coloration in the center measurement was not significant enough to reject the null ($P=0.064$). This phenomenon is likely due to minimal coloration in this area on all frogs, even in parental wild groups.

Discussion:

After feeding Tiger Leg Tree Frogs prey items maintained on various concentrations of broad spectrum carotenoids for several months post metamorphosis, we were able to significantly affect the orange coloration. We were able to show that, like Red Eye Tree Frogs, coloration is very closely tied to carotene intake. Coloration in this study showed dramatic improvement the over control group and even parental coloration.



As a result of *P. tomopterna's* ability to process crickets maintained on high carotene diets, we would also expect that Tank C (test group) to have better long term welfare and reproductive success as implied by other studies (Dugas 2013). Additionally, we would also expect to see that the Tanks B and C would have higher liver Vitamin A levels as a result of beta-carotene present in both diets (Li et al, Odum et al). However, in order to test this, animal would need to be sacrificed. In the event an animal from any of these groups were to expire, liver analysis should be completed to confirm vitamin A levels are indeed appropriate.

The second clutch of *P. tomopterna* was found to be very weak at metamorphosis and had no long-term survivorship. This clutch, as with the first clutch, was split into each tank evenly. Because all individuals from this group expired, regardless of which tank they were exposed to, we can be confident that carotenoid supplementation had nothing to do with poor survivorship. Since all animals from the second clutch did not survive to the time of measurement, they are not represented in this study.

This study helps add to the growing list of positive impacts carotenoid supplementation can have in feeder diets. As with other studies, we have found that *P. tomopterna* also benefits from prey maintenance of carotenes. More research should be done with other species as carotenoids appear to be a silver bullet for many captive amphibian disorders. Most research currently has focused on anurans; however future research should be done to test the class-wide impact carotenes have in prey items.

Implications to Best Practices: In recent years, supplementation of carotenoids has grown in popularity when raising amphibians in captivity. As stated previously, there are many benefits of supplementing carotenoids in feeder diets including increased reproductive success, coloration and Vitamin A levels. Although we are seeing much evidence that carotenoid supplementation appears to improve *coloration*, most welfare benefits appear to be a result of the increased Vitamin A levels associated with carotenoid supplementation. There is evidence suggesting that amphibians are unable to obtain vitamin A from direct ingestion of Beta-carotene (Wright 2006) though. There is also evidence that direct supplementation of usable retinols may have a negative impact on captive amphibians' welfare (Crawshaw 2003). This creates a problem when developing diets to include vitamin A. It is important to note that invertebrates *do* possess the ability to cleave beta-carotene into usable retinol molecules (most notably in adult invertebrate stages that possess a compound eye such as *Drosophila* and *Acheta* spp). As shown by Toledo Zoo, feeding invertebrates a diet (carrots, yams, kale) high in carotenes have

improved liver retinols significantly, showing the ability of invertebrates to convert carotenes into usable forms of retinol. Thus, a prey *maintenance* diet high in carotenes (including beta-carotene and other broad spectrum carotenoids) should be used instead of simply gut loading or direct supplementation of beta-carotene or usable retinols to amphibians. By *maintaining* feeder invertebrates on high carotene based diets, feeders have time to synthesize appropriate retinol levels that would normally be present in wild invertebrates. It is possible that feeder items would also halt production of retinols once dietary requirements are fulfilled, which reduces the chance of Hypervitaminosis associated with direct retinol supplementation. Finally, residual carotenes in feeder items would be utilized to improve coloration of amphibians, as shown in this and other studies.

This study also shows there may be calculable carotenoid concentration appropriate for maintenance diets. Dugas (2013) showed significant improvement in reproductive success at 0.159 - 0.229mg/g of additional carotenoid in fruit fly media, while Ogilvy (2012) showed improvement in coloration of Red Eye Tree Frogs in Post metamorphic diet at 0.25mg/g. This study's Test diet (1.058 mg/g of cricket diet) had significantly lower standard deviation, and restored coloration to that of wild type animals. This suggests that all dietary requirements for carotenoids were fulfilled. 1.058 mg/g of supplemented carotenes in feed items appears to be an adequate feed concentration to guarantee appropriate coloration. However, supplementation at this level may be unnecessary and expensive.

Although orange measurements were higher in the Trace group (0.132 mg/g) than that of the control, the individuals were still significantly less orange and standard deviation was higher in this group. Only one individual in the test group possessed an orange index (9.33) close to that of the test group average (10.21), yet this individual's index score was still lower than *all* animals in the test group. This suggests that the maintenance of crickets on 0.132 mg/g of cricket feed is not sufficient.

There are likely diminishing returns for carotenoid supplementation as indicated by the numbers. Therefore, it is recommended that maintenance diets should be formulated somewhere between 0.132 mg/g and 1.058 mg/g (closer to 1.058mg/g). Further examination of dietary concentrations should be completed to confirm ideal concentrations in order to fulfill dietary requirements while keeping costs to a minimum.

Produce such as sweet potatoes, carrots and kale are commonly used as an alternative to synthetic or algae based carotenoid sources. This allows a nutritious source of carotenoids while also providing moisture for feeder crickets. Although shown to be effective at increasing liver retinol levels in Bufonids (Odum et al), concentrations of these vegetables may not be enough to fulfill carotenoid coloration requirements of Phyllomedusids such as Tiger Leg Tree Frogs and Red Eye Tree Frogs. Sweet Potatoes (0.085 mg/g), carrots (0.083 mg/g) and kale (0.059 mg/g) (USDA) concentrations fail to surpass concentrations of this study's Trace carotenoid concentrations (0.132mg/g). As a result, it may be advisable to consider denser sources of carotenoids for maintenance diets when considering coloration.

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