AN OVERVIEW OF THE DEVELOPMENT AND EVALUATION OF A GUT-LOADING DIET FOR FEEDER CRICKETS FORMULATED TO PROVIDE A BALANCED NUTRIENT SOURCE FOR INSECTIVOROUS AMPHIBIANS AND REPTILES

Lydia Attard
Curatorial Keeper of Invertebrates, Wildlife Animal Care, Toronto Zoo
Toronto Zoo, 361A Old Finch Avenue, Toronto, Ontario, M1B 5K7, Canada

In captivity the diversity of prey items for obligate insectivores is limited and nutritionally inadequate, leading to nutritional deficiencies and consequent diseases (Allen and Oftedal 1982; Bernard *et al*. 1991; Allen *et al*. 1993, 1996; Bernard and Allen 1997; Barker *et al*. 1998; Anderson 2000; Finke 2003; McClements *et al*. 2003; Pessier 2007; Hunt Coslik *et al*. 2009 Pessier 2007 Pessier 2007; Li *et al*. 2009). Zoological institutions utilize gut-loading, a commonly used insect supplementation technique (Allen and Oftedal 1989; Finke *et al*. 2005; Hunt Coslik *et al.* 2009). It essentially offers a nutrient-dense diet to a prey species. This diet is comprised of nutrients which compensate for known inadequacies in the prey's whole body nutrients. As a result, after sufficient ingestion, the insect's gastrointestinal tract (crop and midgut) is filled (Figure 1) and hence, its nutritive composition is comprised of both the insect's whole body nutrients and the retained diet within its gastrointestinal tract. Dependent on the diet formulation, therefore, nutritionally, the gut-loaded insect is expected to be balanced with respect to the nutritional requirements of the end consumer (Allen and Oftedal 1989; Hunt *et al.* 2001; Finke 2003; Finke *et al*. 2005).

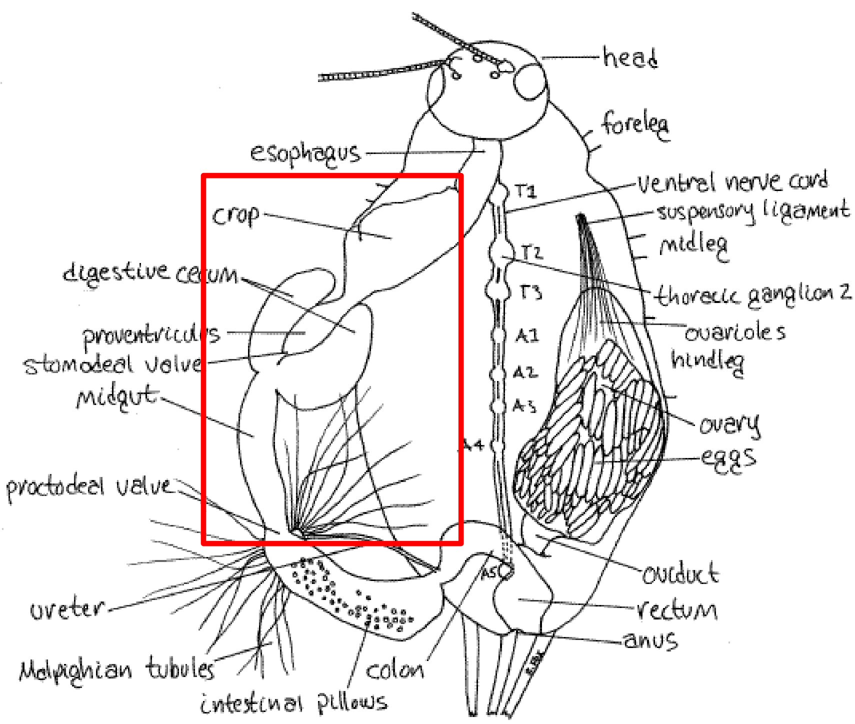

Figure 1 Cricket Digestive System -crop and midgut within the outlined area (Fox, 2001).

Commercially available gut-loading diets, however, have been inconsistent in their success which in part may be due to the diets themselves in that some have been found to fall short of their advertised nutrient levels (Finke *et al*. 2005). Another major contributor may be attributed to the feeder insect's inability to consume the diet appropriately which may be due to the diet and/or perhaps the inconsistent methodologies employed while gut-loading. Consequently, a study was conducted to develop a new gut-loading diet (GLD) to enhance the nutritive quality of one of the most common, commercially available feeder insect species, the domestic house cricket (*Acheta domestica*) Allen and Oftedal 1982; Barker *et al*. 1998; Anderson 2000;

McClements *et al*. 2003; Hunt Coslik *et al*. 2009). In addition to the new diet formulation, a cricket gut-loading protocol was also developed.

The diet was formulated to supplement a 1.67 cm, ~0.23 g cricket, having an estimated 12% gut-loading capacity for insectivorous amphibians and reptiles. The gut-loaded cricket is our nutrient vehicle, delivering all the required nutrients to the end consumer. Consequently, we are working within the restrictions posed by the cricket's nutrient composition and perhaps more importantly by the cricket's gut-loading/consumption ability.

DIET DEVELOPMENT

The development and assemblage of the new GLD involved the selection of nutrients/ingredients based on two objectives. The first objective was to ensure that the mixture of ingredients incorporated delivered sufficient nutrient variety and amounts in physically and chemically absorbable forms to offset any inadequacies in the nutrient value of the cricket body. The second objective was to create a GLD which would optimize cricket ingestion and hence, maximize gut-loading.

Initially cricket nutrient inadequacies were established by first obtaining cricket nutrient compositional values through nutrient analyses performed on fasted cricket samples. These were then compared to those obtained by other investigators (Table1). Insectivorous amphibian and reptile requirements were then determined (Table 2). Amphibian requirement information, however, is very sparse. As a result, based on recommendations made by Finke (pers. comm., September 2005) and other researchers (Hunt Coslik *et al*. 2009), it was assumed that the estimated nutrient requirements for both insectivorous amphibians and reptiles are similar and hence, were mainly based on estimates determined for reptiles. These requirements were established through the comparison of values obtained by various nutritional and medical researchers. However, for some nutrients these are also not well known and were extrapolated from the National Research Council (NRC) requirements for rats and the Association of American Feed Control Officials (AAFCO) requirements for cats (Bernard *et al*. 1991; Allen and Oftedal 1994; NRC 1995; Allen *et al*. 1996; Ferguson *et al*. 1996; Finke 2002, 2003; Abate 2003; Allen and Oftedal 2003; Hunt Coslik *et al*. 2009; Li *et al*. 2009; AAFCO 2010). The nutrient requirements of insectivorous amphibians and reptiles were than compared with the nutrient values of the fasted crickets and the deficiencies calculated.

Basically, crickets are low in calcium (Ca), vitamins A, D and E. Other researchers have also reported low levels of the vitamins B1 and B6, as well as linoleic and linolenic fatty acids (Allen and Oftedal 1994; NRC 1995; Ferguson *et al*. 1996; Finke 2002, 2003; Abate 2003; Allen and Oftedal 2003; AAFCO 2010). These results, and the estimated 12% cricket gut-loading capacity based on a dry matter weight, were then used as a baseline from which to design the new GLD. Its formulation, therefore, would include appropriate levels of the established deficient nutrients.

Consequently, by determining cricket nutrient inadequacies with respect to insectivorous amphibian and reptile requirements, as well as through the assessment of nutrient profiles of various cricket diets and supplements, the proximate component, fibre fraction and specific nutrient ranges were established (Table 3). These were used as bench marks for comparison to either accept or reject the ingredient being evaluated. The ranges were also used as a guideline to design the diet such that the ingredients selected would be incorporated to attain the determined nutrient ranges.

Table 1 Cricket nutrient composition.

Cricket Nutrients	1.67 cm Cricket [a]	1.67 cm Cricket [b]	Nymph Cricket [c]	Nymph Cricket [d]	Nymph Cricket [f]	Adult Cricket [c]	Adult Cricket [e]	Adult Cricket [f]
% DM	26.8			29.9			31.0	
% Moisture	73.2	76.5	77.1		66.8	69.2		73.2
% CP	67.1		67.2			66.6	64.9	
Arginine			4.10			4.06		
Met+Cys			1.44			1.53		
% Ca	0.08		0.12	0.18	1.29	0.13	0.14	0.21
% P	0.72		1.1	0.86	0.79	0.96	0.99	0.78
Ca:P	**0.11**		**0.11**	**0.21**	**1.63**	**0.14**	**0.14**	**0.27**
ADF	9.3		9.6		0.6 (ADF-N)	10.2	9.4	0.7 (ADF-N)
NDF					16.4	22.08		19.1
% Crude Fat	20.4		14.4	17.3	9.8	22.1	13.8	22.8
Linoleic			4.80			7.44		
Linolenic			0.18			0.19		
Vitamin A IU/kg		**51.5**	**140**		**471**	**240**		**811**
Vitamin D3 IU/kg						**831.17**		
Vitamin E IU/kg			**41.7**		**71**	**64.07**		**81**
Vitamin B1 mg/kg			**0.8**			**1.2**		
Vitamin B6 mg/kg			**7.6**			**7.5**		

[a] Three 60 g fasted cricket samples (average weight 0.21 g) analyzed at Daco Laboratories Ltd.
[b] Two 185 g fasted cricket samples analyzed at the Diagnostic Center for Population and Animal Health Michigan State University
[c] Adapted values (Finke 2004)
[d] Adapted values (Allen and Oftedal 1994)
[e] Adapted values (Bernard and Allen 1997)
[f] Adapted values (Barker *et al*. 1998)
Bolded values indicate this study's focus nutrients

Table 2 Recommended Nutrient Requirements.

Recommended Nutrient Levels (DM)	Captive Carnivorous Reptiles [a]	NRC [b]	AAFCO [c]	Reptiles [d]	Green Iguana [e]	Panther Chameleon [f]	Chameleon [g]
% CP	30 – 50	16.67	30.0		26		
Carbohydrates	<20						
%Ca	**0.8 – 1.0**	**0.56**	**1.0**	**3.2-5.1g/kg**	**1.1**		
%P	**0.5 – 1.0**	**0.33**	**0.8**		**0.6**		
% Crude Fibre					6 – 10		
% ADF					10 – 18		
% Crude Fat	10 – 15	5.56	9.0		3		
% C18:2 n-6 Linoleic	**1**	**0.67**	**0.5**		**1**		
Vitamin A (Preformed Vit A - retinyl acetate) IU/kg	**5000 – 10,000**	**2555.5**	**9000 – 750,000**	**2086.9 - 3290.7**	**8000**	**6,500 – 12,350**	**6,500 – 12,350**
Vitamin D3 (Cholecalciferol) IU/kg	**500 – 1000**	**1111.1**	**750-10,000**	**912 – 1440**		**6,500**	
Vitamin E IU/kg	**100 – 300**	**30**	**30**	**25.03**	**150**		
Vitamin B1 mg/kg	**1-5**	**4.44**	**5.0**	**2.5-3.7**	**5.0**		
Vitamin B6 mg/kg	**1-4**	**6.67**	**4.0**	**1.2-2.4**	**6.0**		

[a] Adapted values (Allen and Oftedal 1994)
[b] Adapted values on a DMB based on 10% moisture (NRC 1995)
[c] Adapted values (AAFCO 2010)
[d] Adapted values (Finke 2003)
[e] Adapted values (Allen and Oftedal 2003)
[f] Adapted values (Ferguson et al. 1996)
[g] Adapted values (Abate 2003)

Twenty-two ingredients were evaluated for possible diet incorporation. Some were ruled out because of compositional limitations after completing ingredient nutrient assessments via analysis, as well as through a review of available amino and fatty acid book values (Raw Material Compendium (RMC) 1994). However, most were eliminated because they did not meet the second objective. This was assessed by performing palatability ingredient experiments comparing the amount of each test ingredient consumed. Through these investigations, four ingredients met both selection objectives in that they were determined to be highly palatable and provided most of the necessary nutrients at appropriate levels when combined. Three of the ingredients were plant based. The fourth component was aquatic-plant and marine based. This fourth component was incorporated mainly to provide an appropriate supply of essential fatty acids (EFAs) and other nutrients such as carotenoids which could be beneficial to the end consumer. The formulated diet offers many improvements over other diets mainly due to the plant based ingredients included. The rationale behind this was to offer nutrients to the cricket which would be similar in nutrient profile to the food it would consume in the wild. This would hopefully improve palatability and thereby, maximize consumption (Hunt *et al*. 2007). In addition, the plant based diet could also supply some vitamin A precursors, i.e., carotenoids, and perhaps other nutrients which may also be similar to the natural diet of the end consumer, especially for those consuming mainly herbivorous/omnivorous insects (Hunt *et al*. 2007; Li *et*

Table 3 Proximate components, fibre fractions and specific nutrient ranges for ingredient acceptance and diet formulation.

Component	Acceptance Levels
CP	<20% or 18-24%
Crude Fat	3-6% (low levels recommended to prevent them from meeting their energy requirements before gut loading sufficiently)
α - Linoleic (18:2 n-6)	1%
α - Linolenic (18:3 n-3)	0.03 – 1.39%
Crude Fibre	5-9% (<10% is similar to other cricket diets)
ADF	<9%
NDF	<9%
CHO	45% (many cricket GLD diets are between 40%-62%)
Ca	7%
Vitamin A	8000< IU/kg
Vitamin D	1000 – 50,000 IU/kg
Vitamin E	2000 IU/kg
Vitamin B1	5-31 mg/kg
Vitamin B6	4-50 mg/kg

al. 2009). It may also prove beneficial to include some of the carotenoids in future diet formulations designed for brightly coloured frog species (Ogilvy *et al*. 2012).

In addition to the four primary dietary ingredients, other nutrients, including Ca, vitamin A, D, E, thiamine (B1), and pyridoxine (B6) were also incorporated in an attempt to meet the nutrient requirements of the insectivorous amphibians and reptiles. The vitamins were added via a premix, based on general insectivorous amphibian and reptile requirements. However, specific premixes can be prepared for insectivores whose requirements are subsequently found to differ from the generic values used. The diet base can be used with a variety of vitamin premixes allowing nutrient flexibility with respect to the formulation of the GLD for the end consumer.

A second diet without any added Ca was also formulated. This low Ca insect diet was used as a control in the experiments and helped establish that there was a decrease in consumption by the crickets feeding on the GLD most probably due to its decreased palatability as a result of the high Ca levels in the diet. It also determined that there was no increase in cricket mortality for the duration of the experiments by the crickets consuming the GLD. However, the high calcium GLD is not intended to provide long-term insect nutrition and its prolonged use may lead to insect mortality. Consequently the low Ca insect diet will also be fed to sustain, rather than gut-load, the zoo's captive herbivorous insect colonies e.g., cockroaches (Blattodea), etc. which in some cases may then be used as feeder insects for various predatory insectivore invertebrates (Allen and Oftedal 1989; Bernard and Allen 1997; Hunt *et al*. 2001; Finke *et al*. 2005).

GUT-LOADING OPTIMIZATION

Gut-loading success is contingent on optimizing the cricket's ability to ingest the supplementation diet. This was accomplished by ensuring that the physical form and the nutritive provisions of the diet met with the foraging and palatability requirements of the vehicle of nutrient delivery, the cricket. Gut-loading/consumption constraints include the physical form of the diet, i.e., particle size. This was considered early on in the diet formulation so as to ensure the diet's optimal ingestion. Crickets, in general, prefer finely ground diets since the smaller size facilitates and maximizes cricket consumption, especially by emergent and developing crickets in that their small mouth parts are accommodated (Allen and Oftedal 1989; Bernard and Allen 1997; Anderson 2000; Finke 2003; Finke *et al.* 2005). As a result of diet size experimentation, the diet has been finely ground to small particles sizes (0.212 mm - 1 mm) similar to flour in consistency. In the future, due to the diet consistency, it could also be used as a "dusting agent" to coat the crickets, prior to feeding.

Another constraint is palatability, a function of the amount and quality of nutrients. Diet (nutrient/ingredient) palatability is one of the most important, affecting cricket ingestion optimization and gut-loading success. Accordingly, this was accounted for during the formulation of the diet and hence, the nutrients/ingredients incorporated were not only added to offset the cricket's inadequate nutrient composition but also to compensate for any consumption limitations. For example, the possible effect of poor palatability was countered through the selection of highly palatable ingredients.

There are many factors besides the diet's physical form and palatability which influence a cricket's optimal gut-loading ability and if these are not taken into consideration during cricket feeding than the success of the gut-loading nutrient supplementation technique may be compromised. Consequently, the methodologies employed throughout this study's experimentation have been inclusive of various factors which optimize cricket ingestion. Many of these have been identified through the reports and investigations of other researchers (Allen and Oftedal 1989; Allen 1997; Bernard and Allen 1997; Anderson 2000; Hunt *et al.* 2001; Finke 2003; Finke *et al.* 2005). Consequently, cricket behavioural and physiological factors (e.g., size/gut fill capacity), as well as their environmental requirements were standardized and implemented throughout the experiments.

Cricket age and size (gut-loading capacity) were two such factors. Crickets in their last week of life do not feed readily (Trusk and Crissey 1987), consequently, nymphs (of a specific size) were used for each of the experiments. On a dry matter basis the gut-loading capacity as a percentage of body weight decreases with increasing body size (Hunt *et al.* 2001; Finke 2003; Finke *et al*. 2005). According to Finke (pers. comm., September 2005), adult crickets have an 11-13% capacity while nymphs have 13-15%. Consequently, for a 1.67 cm cricket we based the diet on a 12% gut-loading capacity.

The moisture source was another factor considered. For this study a constant provision of distilled water as opposed to using produce or other types of moisture sources was chosen. A decision confirmed by many investigators, since water has been considered an important factor in promoting food consumption (Trusk and Crissey 1987; Bernard and Allen 1997; Ward and Crissey 1997). This is especially true for diets that are high in dry matter and contain high levels of Ca since crickets must maintain their moisture levels. In addition, due to the poor palatability of high Ca diets, produce offered as a water source may be consumed in preference to the actual diet (Allen and Oftedal 1989; Bernard and Allen 1997; Ward and Crissey 1997). Consequently, distilled water was used in the experiments (rather than tap water) so that no additional nutrients were consumed by the crickets.

Additional factors include temperature and humidity ranges. Low temperatures cause reduced feed intake resulting in sub-optimal Ca:P ratios (Ward and Crissey 1997). In contrast, high temperatures cause an increased rate of development (Ghouri and McFarlane 1958; Castner 2000). Crickets prefer temperatures between 26-32°C (Finke *et al*. 2004). Other researchers suggest that temperatures between 28 and 35°C are optimal (Ghouri and McFarlane 1958). All experiments in this study were carried out with temperatures ranging from 26 to 29°C at the bottom of the experimental tanks and 29 to 34°C at the top of the egg cartons. Consequently, a temperature gradient was provided with higher temperatures at the top of the tank that the crickets could access by being in the top portion of the egg cartons. This was important as the crickets could choose their optimal temperature.

Ghouri and McFarlane (1958) reported that for the domestic house cricket (*Acheta domestica*) the relative humidity should be kept low, around 50%. This drier environment was chosen since it was the lowest at which no clustering of the insects around the water source was observed and it more closely approximated the natural conditions under which the insect lives. There was also no danger of food spoilage (Ghouri and McFarlane 1958). In fact, the relative humidity for most experiments in this study was below 40% and usually 20% with no ill effects observed. This range was selected as humidity levels between 30 and 50% decrease the reproduction of dust mites, mould, bacteria and airborne viruses (Arundel *et al.* 1986).

Other factors were discovered inadvertently during the course of the study. One was the significance of positioning feed dishes near sheltered areas to increase ingestion. Researchers working with rats have found that they, like many other animals, exhibit wall seeking behaviour in that they tend to restrict their activity to sheltered areas, moving along edges, rather than out into the open (Brubaker 1975). Crickets also appear to exhibit this trait. Much of the experimentation demonstrated that the contents of the dishes positioned by sheltered areas, which afforded more secure conditions, were generally found to be better consumed than those which were in more open locations (Table 4). Statistically during the first 26 hrs, the contents of the dish in position #5 were consumed more than the similarly consumed contents of the dishes in positions #1, #4 and #3 with that of #2 being the least preferred. Therefore, the expectation that the dishes in positions #1 and #5 would have similar and higher consumption amounts than those of the three middle dishes was not observed. This may have been due to their appetite which was probably increased during their shipment and was at elevated levels during this time period and hence, their motivation to eat outweighed their drive to seek out secure locations. At the 50 hr time period the data show a significant preference for the contents of the dish in position #5 and even that of the dish in position #1 although there is some statistical similarity between the consumption amounts of the contents of the dishes in positions #1 and #4 with the contents of the dishes in positions #3 and #2 being least preferred. At this 50 hr time period the crickets were probably more focused and less motivated by hunger and hence, security once again became a priority. Even though the contents of the dishes in positions #1 and #5 were expected to be consumed in preference to the three middle dishes, the dish in position #4 for all the time periods following the first 26 hrs was consumed to the same degree as that of the dish in

Table 4 Consumption amounts of ingredient L to illustrate importance of food dish placement.

CONSUMPTION	~26 hrs	~50 hrs	~74 hrs	~98 hrs	~122 hrs
Dish Position	Average Consumption (g)	Average Consumption (g)	Average Consumption (g)	Average Consumption (g)	Average Consumption (g)
1	7.5 ^{a}	11.3 ^{a}	14.2 ^{a}	15.8 ^{a}	17.2 ^{a}
2	4.8 ^{b}	6.8 ^{b}	8.5 ^{b}	9.7 ^{b}	11.1 ^{b}
3	5.4 $^{a, b}$	7.8 ^{b}	9.8 ^{b}	10.8 ^{b}	12.1 ^{b}
4	6.5 $^{a, b}$	9.0 $^{a, b}$	11.2 $^{a, b}$	12.0 $^{a, b}$	13.2 $^{a, b}$
5	11.1 ^{c}	16.5 ^{c}	20.0 ^{c}	24.8 ^{c}	27.3 ^{c}
Avg. Total Intake (g)	35.4	51.3	63.7	73.1	81.1

At each time period different superscripts indicate significant differences between consumption amounts ($P<0.05$).

position #1 with the contents of the dishes in positions #3 and #2 being the least preferred. However, upon closer inspection it was discovered that the dish in position #4 was in a shadow thus affording it more security. In addition, the dish in position #4 was the closest to the dish in position #5 the location at which the contents were the most consumed. As a result, this proximity may have had an additional effect, causing more crickets to visit and consume the contents of this dish on their way to the dish in position #5 and due to the shadow, they remained consuming its contents because of the security it offered. The data show a similar trend as observed at 50 hrs for succeeding time periods, however, a preference for the contents of the dish in position #1 over that of the dish in position #4 becomes more evident as time passes. Consequently, the cricket's natural behaviour to seek out secure locations can be employed to increase the comfort level of the animal and thereby, provide more security where the food is offered. As a result, the benefits of maximum gut-loading would be more likely realized.

Another factor discovered during the experimentation was the importance of the cricket’s developmental stage within its instar in that optimal consumption occurs during the growth phase of the instar (Ghouri and McFarlane 1958; Woodring 1983; Daly *et al*. 1998). Virtually all hemimetabolous insects develop from an egg to an adult by passing through a series of stages called instars. Crickets will go through 6-8 instars before becoming an adult (Ghouri and McFarlane 1958). As they go from one instar into another they moult, shedding their exoskeleton. Optimal food consumption occurs during the growth phase of the instar (Ghouri and McFarlane 1958; Woodring 1983; Daly *et al*. 1998). Growth occurs between moults. Crickets begin feeding about 6 hours after moulting and continue until mid-instar (Figure 2). During this time they feed every couple of hours filling portions of their digestive system such that they maintain a distended crop and a full midgut. After they reach this mid instar and as long as they have achieved their critical weight gain, they stop feeding and the moulting process begins (Woodring 1983). These abstinence periods could have a negative effect on gut-loading success,

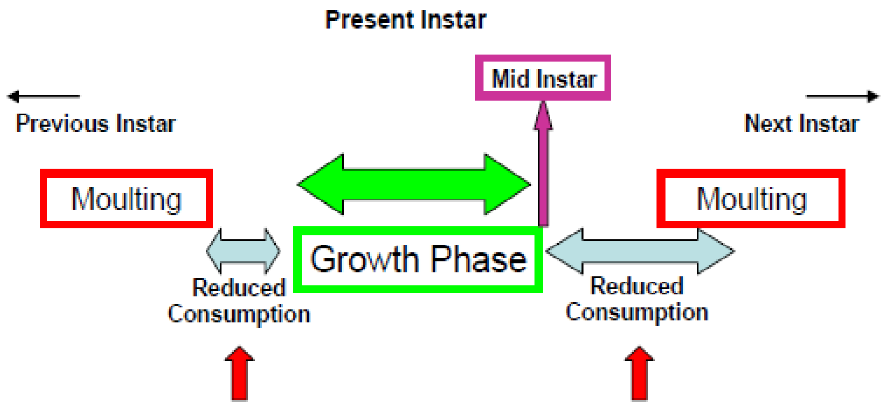

Figure 2 Stages of development within the cricket instar. Ingestion of food and water only occurs during the growth phase.

especially as the cricket reaches the end of the instar. Consequently, the decision as to whether or not crickets were used in an experiment was dependent on this stage of development. Basically only those within the growth phase were used. Generally, this was established over the first 24 hours after a cricket delivery using two indicators. The first was the amount of sheds in the experimental tanks. Many sheds (M) indicated that many crickets were moulting and were at the end of an instar (E), while few sheds (F) indicated that many were in a growth phase (G). The other indicator used was the amount of diet consumed, compared to a previously established maximum consumption level. This diet palatability experiment (Table 5), therefore, illustrates the significant effect of the developmental stage on consumption. The crickets used in treatments 1A and 1B had many sheds (M) in the debris indicating that most of the crickets were probably at the end of an instar (E). This was confirmed by the low levels of diet A consumed over a 24hr period for each of these two experiments, i.e., less than 65% compared to the consumption of the previously established maximum potential benchmark consumption level. This was also confirmed by the low levels of each diet consumed for each of these experiments compared to the consumption amounts of the respective diets in treatments 1C and 1D. Most of the crickets used in treatments 1C and 1D were in a growth phase (G), as indicated by the low number of sheds (F) in the debris. Their diet A consumption levels were also comparable to the consumption amounts of the previously established maximum potential benchmark consumption level indicating that diet A had been sufficiently ingested for these experiments. The other diets were also consumed adequately since they were consumed within at least 65% of the maximum potential benchmark consumption level. It should also be noted that the crickets of treatment 1D had been additionally fasted for 24hrs after delivery before the diets were offered to increase ingestion. As a result, this additional fasting period may have contributed to the desired effect in that the crickets of treatment 1D had much higher consumption levels. Consequently, treatments 1C and 1D illustrated that the new GLD was able to be sufficiently consumed provided that the crickets were in the growth phase of their instar.

Table 5 Cricket weights and diet consumption amounts to determine diet palatability.

Tanks	1.5.9				2.6.7				3.4.8				
Diet	A (Crushed Rabbit Pellets)				B (Low Calcium Insect Diet)				C (High Calcium GLD)				Max. potent. benchmark consump. (Ingredient L)
Treatment	**Trtmt 1A**	**Trtmt 1B**	**Trtmt 1C**	**Trtmt 1D**	**Trtmt 1A**	**Trtmt 1B**	**Trtmt 1C**	**Trtmt 1D**	**Trtmt 1A**	**Trtmt 1B**	**Trtmt 1C**	**Trtmt 1D**	**Trtmt 3-1**
Avg. Fasted Cricket Wt. (g)	**0.24**	**0.21**	**0.24**	**0.18**	**0.24**	**0.21**	**0.24**	**0.18**	**0.24**	**0.21**	**0.24**	**0.18**	0.21
Stage of Development within its instar †	**E, M**	**E, M**	**G, F**	**G, F**	**E, M**	**E, M**	**G, F**	**G, F**	**E M**	**E, M**	**G, F**	**G, F**	
Avg. Cricket Wt. (g) after feeding on diet for 2 days	**0.26**	**0.25**	**0.29**	**0.24**	**0.28**	**0.26**	**0.30**	**0.27**	**0.27**	**0.26**	**0.30**	**0.27**	
Avg. Fasted Cricket Wt. (g) after feeding on diet for 2 days	**0.24**	**0.22**	**0.27**	**0.24**	**0.25**	**0.24**	**0.27**	**0.25**	**0.24**	**0.22**	**0.26**	**0.23**	
Avg. Wt. (g) loss due to retention?	**0.02**	**0.03**	**0.02**	**0**	**0.03**	**0.02**	**0.03**	**0.02**	**0.03**	**0.04**	**0.04**	**0.04**	
Avg. Consump. (g) after ~24 hrs Debris Avg. (g)	**23.3** ^{a,1} Debris – 20.5	**25.3** ^{d,1} Debris – 28.4	**47.9** ^{f,2} Debris – 27.9	**61.1** ^{i,3} Debris – 33.8	**18.9** ^{b,4} Debris – 20.3	**20.2** ^{e,4} Debris – 26.6	**40.4** ^{g,5} Debris – 18.2	**55.5** ^{ij,6} Debris – 22.3	**16.1** ^{c,7} Debris – 22.9	**19.5** ^{e,7} Debris – 24.8	**34.0** ^{h,8} Debris – 19.2	**50.9** ^{j,9} Debris – 24.8	**47.35***
Avg. Cumul. Consump. (g) after ~48 hrs Debris Avg. (g)	**38.6** ^{k,10} Debris – 21.9	**51.4** ^{n,11} Debris – 27.2	**78.5** ^{p,12} Debris – 29.7	**107.2** ^{s,13} Debris – 38.7	**32.1** ^{l,14} Debris – 17.5	**41.3** ^{o,14} Debris – 20.1	**66.0** ^{q,15} Debris – 21.0	**103.7** ^{st,16} Debris – 26.7	**26.3** ^{m,17} Debris – 18.2	**37.6** ^{o,18} Debris – 20.5	**56.6** ^{r,19} Debris – 21.2	**93.1** ^{t,20} Debris – 25.1	**72.49**

* Adjusted from 26hrs consumption to 24hrs consumption
L (Crushed Rabbit Pellets)
† E – possible instar end (many sheds (M)), G – possible growth phase (few sheds (F))Different superscripts indicate significant differences ($P<0.05$). Alphabetical superscripts indicate dietary consumption comparisons within treatments at 24 hrs and at 48 hrs numerical superscripts indicate dietary consumption comparisons across treatments at 24 hrs and at 48 hrs.

The decline in ingestion by crickets at the developmental end of an instar may explain some of the inconsistent gut-loading success experienced by many researchers (Allen and Oftedal 1982; Trusk and Crissey 1987; Allen and Oftedal 1989; Anderson 2000; Hunt *et al.* 2001; McClements *et al*. 2003; Finke 2004; Finke *et al.* 2005; Hunt Coslik *et al.* 2009). This has many implications since if most of the crickets delivered by the supplier are at the end of their instar they will not have ingested the gut-loading diet sufficiently and their potential as a vehicle to deliver nutrients to the end consumer would not be fulfilled.

Accordingly, many of these factors, i.e., physical form of the diet, diet palatability as well as the behavioural, physiological and environmental factors mentioned were accounted for during the GLD formulation. They were also used to develop cricket gut-loading optimization recommendations to ensure the production of an optimal nutrient package and hence, a high quality diet. These cricket gut-loading optimization recommendations have been listed as a companion to the GLD (Appendix Table 1).

DETERMINING GUT-LOADING SUCCESS

Although the diet's degree of palatability was assessed by following the gut-loading optimization recommendations and ensuring that it had been sufficiently consumed when compared to the maximum potential consumption benchmark there was still some uncertainty as to whether the diet had been successfully retained and hence, that the crickets had successfully gut-loaded. The final step in the evaluation process to assess the effectiveness and success of this new GLD was ultimately verified by determining the nutrient composition of gut-loaded crickets over time. This established the length of time required for them to optimally gut-load and measured how long the benefits of gut-loading last. This confirmation ensured that the weight gained through diet consumption was due to its appropriate retention in the GIT and not just to tissue development or water retention.

This set of experiments also investigated the effect of hunger on consumption and hence, when gut-loading should begin. Previous experiments indicated that crickets consume comparatively more of the food offered during the first 24 hrs after delivery than during any other time period. This increase in consumption may be a useful tool to optimize ingestion. Delivered crickets arrived in some semi-fasted condition due to being shipped with a small number of potatoes or carrots (included as a moisture source). Consequently, without their normal diet and the stress incurred during shipment, a state of starvation exists, the severity of which increases depending on the duration of the shipment. It appears that when crickets were offered food after undergoing some degree of "fasting" their consumption increased and this maximized their gut-loading potential. Consequently, for some of the experimental treatments the crickets were fasted for a further 24 hrs after delivery to increase consumption and optimize their gut-loading ability.

The crickets used in treatment 2A were in a growth phase as indicated by the low number of sheds in the debris and their consumption amounts which were comparable to the established maximum potential benchmark consumption level (i.e., over the 65% minimum level after gut-loading for 24 hrs) (Table 6). In order to maximize gut-loading, these crickets had also undergone a further 24 hr fasting period, in addition to the one experienced during shipment before the GLD was offered. The target minimum Ca:P ratio was achieved after they had been gut-loaded for only one day, reaching a maximum level after two days and although it did decrease over the next two days, the levels were still above the minimum.

The target linoleic value was exceeded even before they began gut-loading. This indicated that the crickets had this nutrient as part of their whole body nutrients. This also concurred with other researcher findings (Finke 2004). The levels then increased over the next few days, indicating effective gut-loading. These increased levels continued even in the fasted crickets which had gut-loaded for four days, suggesting that the fatty acid may have become part of the cricket tissue.

The minimum target vitamin A levels were achieved, again after gut-loading for only one day, and these continued rising until the fourth day at which time they decreased but were still above the target minimum.

The vitamin E levels, however, exceeded our target maximum after gut-loading for only one day. The levels of this vitamin's addition to the diet may be too high in that it may interfere with vitamin A absorption in the end consumer (NRC 1995; Finke 2003). The anti-oxidant ability of this vitamin and its presence at these higher levels may compensate for cricket fat and especially unsaturated fat levels (NRC 1995; Finke 2002). The amounts of vitamin E added need to be re-examined to assess whether they should be altered.

Table 6 Nutrient and consumption amounts to determine gut-loading success.

Experiment	Experiment #2A[a, c]							Maximum Potential Benchmark Consumption Experiment 3-1[b]	Experiment 1D[b, c]		Experiment #2B[a]				Experiment #2C[a]	
Developmental Stage	Growth Phase						Target Amounts		Growth Phase		Growth Phase				Instar End	
Dry Matter Basis	Fasted Cr Avg	Day 1 Avg	Day 2 Avg	Day 3 Avg	Day 4 Avg	Fasted Cr Avg		Day 1 Avg	Day 1 Avg	Day 2 Avg	Day 1 Avg	Day 2 Avg	Day 3 Avg	Day 4 Avg	Day 1 Avg	Day 2 Avg
%Dry Matter	24.2	23.3	24.03	23.9	24.2	24.7	20-30			24.6	23.57	23.17	23.2	22.73		26.567
% Moisture	75.8	76.7	75.97	76.1	75.8	75.3	70-80			75.4	76.43	76.83	76.8	77.27		73.433
% Crude Protein	79.7	75.23	69.23	66.77	65	73.3	60-70			64.667						
% Calcium	0.0917	1.1112	1.5857	1.1338	1.3922	0.3918				1.4152						0.6112
% Phosphorus	1.0392	0.9856	0.9212	0.8175	0.8717	0.8786				0.8446						0.8371
Ca:P	**0.088**	**1.127**	**1.721**	**1.387**	**1.597**	**0.446**	**1.0-1.6**			**1.676**	**1.168**	**1.249**	**1.224**	**1.038**		**0.730**
% Crude Fibre	10.47	9.33	9.5	10.33	8.53	10.5				10.33						
% Crude Fat	11.33	11.27	12.43	15.13	16.3	16.6	10-15			14.5						
% Linoleic	**4.78**	**4.62**	**4.87**	**5.83**	**6.02**	**6.43**	**1**			**5.39**						
% Linolenic	0.0076	0.02	0.034	0.035	0.044	0.03				0.04						
Vitamin A (Retinyl Acetate) (IU/kg)	**<333**	**12607**	**18672**	**29694.7**	**22170.7**	**6202.3**	**8000<**			**20012.7**						**5637**
Beta Carotene (IU/g)	<0.42	<0.42	<0.42	<0.42	<0.42	<0.42	3.19			<0.42						
Vitamin E (IU/kg)	**7.3**	**342.3**	**561**	**516.4**	**527.07**	**185.47**	**100-300**			**502.8**						
Cumulative Consumption Amounts (g)		**44.1** **31.5****	**92.0**	**136.5**	**174.2**			**47.35***	**50.9**	**93.1**	**51.0** **36.4****	**84.1**	**110.0**	**126.7**	**22.5** **16.0****	**34.4**

* Adjusted from 26 hrs consumption to 24 hrs consumption
** Adjusted from 420g 1.67 cm crickets to 300 g
[a] 420 g 1.67 cm Crickets
[b] 300 g 1.67 cm Crickets
[c] Crickets used in this experiment had been fasted for an additional 24 hrs after delivery before food was offered

The beta carotene levels were surprising, as a higher level in the region of 3.19 IU/g was expected once gut-loading began, especially since the analytical values obtained for the GLD averaged 26.57 IU/g on a DM basis. After much discussion with the lab performing the analysis, however, we were still unable to substantiate the gut-loaded cricket analytical results for this nutrient. They remain questionable and hence, this nutrient will require further gut-loading experimentation and subsequent analysis for proper assessment. Other carotenoids may also be included in future analyses since these may be valuable for some brightly coloured amphibians (Ogilvy *et al*. 2012).

The next three treatments (one of which was treatment 1D) were added to reinforce the importance of the cricket's developmental stage to gut-loading success. As mentioned earlier, the crickets used in treatment 1D had undergone the additional 24 hr fasting period after delivery before being offered the diet in order to increase ingestion. The crickets used in treatments 1D and 2B were in a growth phase as indicated by few sheds in the debris and their consumption amounts which were well over 65% of the established maximum potential benchmark consumption level. Both experiments had similar nutrient values to treatment 2A at the two day gut-loading period. Crickets in treatment 1D were only analyzed at this time period due to financial constraints. This time period was chosen based on reports of other researchers who found that cricket gut-loading potential was usually maximized after two days of gut-loading (Allen and Oftedal 1989; Bernard *et al.* 1997; Hunt *et al.* 2001; Finke *et al.* 2005). The crickets of treatment 2B, which had been shipped to the Diagnostic Center for Population and Animal Health for analysis, were delayed during shipping and hence, due to their decomposed condition on arrival, could only be analyzed for moisture, Ca and P. However, the crickets used in this treatment gut-loaded adequately in that their Ca levels were similar to those of treatments 2A and 1D, albeit numerically lower but still above the target values which continued over the four day gut-loading period. Treatments 2A and 1D for which the crickets were additionally fasted for 24 hrs had higher Ca:P ratios than those of treatment 2B which were not additionally fasted. The fact that the analyses performed were on decomposed crickets may also be confounding the results. The effects of fasting on gut-loading, therefore, require further investigation.

The crickets used for treatment 2C were not in a growth phase but at the end of an instar as indicated by the large number of sheds in the debris and the lower consumption, i.e., much lower than 65% of the established maximum potential benchmark consumption amount. The cricket consumption levels were also lower than those of the other three experiments. This was confirmed by the nutrient levels reached after a two day gut-loading period which were below the minimum intended values for the Ca/P ratio and for vitamin A. After two days of gut-loading the nutrient values analyzed were also well below those reached by the crickets in treatments 2A, 1D and 2B, which were in a growth phase after an equivalent period of gut-loading. This emphasizes the point that for gut-loading to be successful, crickets must be in the growth phase of their instar.

In conclusion, the GLD was consumed sufficiently after following the gut-loading recommendations to optimize consumption. This was especially true once the developmental stage of the crickets being gut-loaded had been determined. As a result, the analysis verified the success of the new GLD in that the combination of the cricket whole body nutrients and the diet retained in the gastrointestinal tract contained all the nutrients in the amounts intended (except for vitamin E and beta carotene) with respect to the estimated nutrient requirements of the intended consumers, the insectivorous amphibians and reptiles. These values also illustrated that the target nutrient levels were achieved after gut-loading for only 24hrs (Ca:P of 1.127; vitamin A (retinyl acetate) level of 12,607 IU/kg; vitamin E level of 342 IU/kg and a linoleic fatty acid level of 4.62%). The benefits of this technique were realized over the full four day gut-loading period in that the nutrient levels attained remained above target levels and hence, above the estimated requirements of the end consumers throughout the duration of the experiment.

Consequently, this GLD in combination with the recommended gut-loading methodology achieved more consistent success and was an improvement over other previously tested diets. Researchers who achieved the required nutrient levels were only able to do so for a period between 2-4 days of gut-loading (Allen and Oftedal 1989; Anderson 2000; Finke 2004; Finke *et al.* 2005; Hunt Coslik *et al.* 2009). The crickets consuming the new GLD could be used for feed after one day of gut-loading and could continue to be fed off over a maximum of four days of gut-loading which may be extended with further testing. Continued experimentation is required to examine the new GLD's effects on growth, reproduction and health of insectivorous amphibians and reptiles consuming the gut-loaded crickets.

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Appendix Table 1 Cricket gut-loading optimization recommendations.

Holding/feeding Tanks

- Cricket holding/feeding containers should be set up as close as possible to the animals they will be fed to. This will minimize movement stress and temperature/humidity variation. It will also allow the animals to feed longer, right up until they are moved into the predator's container. As a result an optimum nutrient package would be provided to the end consumer.
- The holding area should be relatively quiet with as little disturbance as possible since loud noises and a lot of activity could decrease consumption.
- Food and water dishes should be offered in sheltered areas – provide cover (i.e. egg flats) over dishes where possible.
- Gut-loading crickets should **never be offered produce** as this basically defeats the purpose of gut-loading. The produce may also have been grown using pesticides which may, therefore, harm the crickets and perhaps their intended consumers. For pinheads to 1/8 crickets offer a 2 day supply of the GLD on the floor of the container to facilitate consumption. Check daily and add additional food as required. For ¼ to adult crickets offer a 2 day supply of the GLD in dishes. Stir the dish contents daily to prevent food particle sorting. After 2 days discard the remaining food and offer another 2 day supply.
- Use distilled water or dechlorinated water ad lib. For ¼ to adult crickets use a 1L water dispenser with pebbles in the trough to prevent drowning and to facilitate cleaning. Cleaning of the water dispenser and its replenishment should be done as required before the water in the trough becomes foul. For very young nymphs i.e. pinhead to 1/8 crickets a green scrub pad (cut to the inside diameter of the dish) should be used to prevent drowning and to facilitate daily cleaning.
- A temperature gradient should be maintained with temperatures of 26-29^{o}C at the bottom of the holding tanks to 29-34^{o} C at the top of the egg flats. These temperatures can be maintained through the use of two 75 watt ceramic heaters/tank and a Helix thermostat whose sensor is located approximately 3" from the middle of the bottom of the tank.
- Relative Humidity should be maintained below 50% and preferably between 20-40%. To decrease humidity increase ventilation by using a fan. For increased humidity decrease air circulation by decreasing air movement via a piece of plastic or cloth over the lids, etc.
- Each cricket holding tank should be equipped with a sufficient amount of egg flats to maximize surface area and minimize cannibalization.
- Clean ¼ to adult cricket holding tanks daily to minimize debris/fecal consumption and to maintain hygienic conditions.
- After 4 days clean out the container and replace the egg flats to have it ready for the next cricket delivery.

Cricket Feeding Suggestions

- The crickets in most cricket shipments have experienced some degree of fasting before they arrive at their destination. The cricket's appetite can be increased by prolonging the duration of fasting for another 24hrs. An additional benefit is that their gut contents will be evacuated such that when the GLD is offered they will retain only the diet and perhaps water in their gastrointestinal tracts and hence, no other nutrients. They may, therefore, achieve a sufficient gut-loading nutrient level earlier than if fasting was not extended.
- If possible, gut-load crickets which are in a growth phase and not at the end of an instar. This could be determined within 24 hrs of delivery dependent on the number of sheds present and the amount of diet consumed. If they are found to be at the end of an instar they should be fed the low Ca insect diet until they enter the growth phase. Once this phase is reached gut-loading can then begin. However, only gut-loading crickets which are in a growth phase may be impractical as it may disrupt predator feeding schedules, as well as the cricket delivery since if crickets are not used within the time allotted, the next delivery would have to be adjusted. Over the course of a weekly predator feeding schedule, however, it may also be unnecessary since the predator will most probably still receive all its required nutrients. If, however, there are animals which have been diagnosed with a nutrient deficient disease then presumably it would be crucial to ensure that the crickets fed to these clinically deficient animals are always optimally gut-loaded. Under these circumstances, therefore, the crickets fed to these vulnerable animals could be set aside from the main group of feeder crickets. One could then ensure that these crickets are in a growth phase before they are gut-loaded and subsequently fed off to the nutrient deficient animals.
- Before offering them to the end consumers allow them to feed on the GLD for at least 1 day and no longer than 4.
- If possible, at the same time everyday collect the crickets to be fed off to the end consumers. Once they have been fed off then clean, stir food dish contents or feed/water the crickets as required. This will minimize cricket disturbance and optimize gut-loading and will result in being able to offer an optimal nutrient package to the end consumers.

Diet Storage and Use

- Once the sealed diet container has been opened, the remaining diet should be stored in a refrigerator/freezer <4^{o}C to minimize nutrient deterioration and used within 180 days of opening. Once the container is half full, the remaining diet should periodically be thoroughly mixed before feeding so as to minimize nutrient separation. Care should also be taken to ensure that condensation does not develop in the container which could induce diet contamination (Allen 1997; Finke 2003).
- A program to assay nutrients should also be implemented to routinely verify the composition of the diet.