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Assessing the performance of *Alphitobius piceus* (Oliver, 1792) as novel feeder insect species for small sized postmetamorphic frogs

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Abstract. One of the major difficulties of frog farming is providing adequate food in the first few weeks after the metamorphosis. This is a critical time frame, with rapid growth and high mortality. The established feeder insect species used throughout this delicate stage (cricket and mealworm larvae, adult fruit flies) are suitable from a trophic perspective, but present challenges and difficulties that influence the production costs. The aim of this study is to assess the performance of an alternative feeder species – *Alphitobius piceus* – with a simpler production technology and a lower production cost. Two species of feeder insect were used: the conventionally used *Acheta domestica* larvae as control species, and *Alphitobius piceus* larvae as experimental species. As model anuran we used the European common frog, *Rana temporaria*. The experimental insect species was easily accepted and produced a strong feeding response in the post-metamophic frogs. There were no significant differences between the control and experimental groups in terms of body condition index and mortality, however survival was better in the experimental group. This similarity indicates a high potential of *Alphitobius piceus* as feeder species for the newly metamorphosed frogs and also a good economic opportunity for frog farms.

Keywords: Frog farming, live prey, feeding response, juvenile frogs.

Introduction

Frog legs are a popular and valuable product; therefore, frog farming becomes one of the most attractive commercial enterprise in aquaculture (Miles *et al.,* 2004). However, before considering setting up an edible frog farm, the problem of constantly supplying a large quantity of live prey must be addressed and resolved. The production of live food becomes, therefore, an activity as important as the production of the frogs themselves. Keeping the cost of this production at the lowest possible level, through a reduced infrastructure and a simple technological process, will be a top priority and a good indicator of the profitability.

Live prey size is also an important problem to address. Providing the right type of food item can be especially problematic in the period right after the metamorphosis, because of the small size of the postmetamorphic animals. This is a critical period for the frogs, when the growth rate (Breckenridge and Tester, 1961; Labanick and Schlueter, 1976) and the mortality (Pechmann *et al.*, 1991; Roznik and Johnson, 2009; Scott *et al*., 2007) is the highest, and they are not yet able to feed on live maggots. At this stage, the animals are commonly fed on adult common fruit flies (*Drosophila melanogaster*), cricket larvae in early stages (*Grillus* sp. *Acheta* sp.) or early-stage larvae of the darkling beetle (*Tenebrio molitor*) (Miles *et al.*, 2004; Culley *et al.*, 2009). All of these feeder species satisfy the trophic needs of the frogs but their production and handling can often prove problematic. For example, cricket farming technology is complex and needs a significant infrastructure (Hanboonsong *et al.*, 2013), the darkling beetle is easy to grow and reproduce, but separating the early instar stage larvae needs a fairly complicated tray stack rearing system (Morales-Ramos, 2012) and the common fruit fly has a high escape capacity. These are apparently small inconveniences but they translate in to higher production costs. Therefore, the identification of a novel, more advantageous feeder insect species for post-metamorphic frogs is a timely enterprise.

The neuro-ethological studies conducted by Ewert (1980) have largely deciphered the nature of the stimuli that trigger feeding behaviour in anurans. Thus, the most appreciated prey shape proved to be the most worm-like, similar to a horizontal line with a strong contrast to the substrate.

In this study we propose to bridge the gap between metamorphosis and maggot eating size frogs, using *Alphitobiul piceus* (the lesser mealworm) – a small sized beetle – as a possible candidate. The species is easy to grow and reproduce and the larvae seem to be suitable as shape (Ewert, 1980) and size (Kuzmin, 1990) (Fig. 2), making it potentially more useful than the species generally used as food in the early post-metamorphic stage.

The purpose of the study is rearing juvenile post-metamorphic frogs in a more cost-effective way by testing the efficiency of the lesser mealworm larvae as experimental food source for post-metamorphic juveniles of *Rana temporaria*, compared with the control feeder insect species *Acheta domesticus*. For a better perspective on the potential advantage of the experimental species, we focused on the resultant growth and mortality rather than the consumption rate of the food items.

Materials and methods

The model anuran

We used the European common frog (*Rana temporaria*) as experiment species. It is a semiaquatic edible species, widely consumed ethno-gastronomicaly throughout Transylvania (Lengyel, 2016) and has a LEAST CONCERN status in the IUCN Red List of Threatened Species. At adult stage, it's food consists of a wide spectrum of terrestrial invertebrates, arthropods and mollusks, the differences of diet preferences between populations being attributed only to the availability of the prey species (Tiberti *et al*., 2015). Consequently, the common frog behaves like a generalist predator, eating all mobile prey types large enough to be observed and small enough to be swallowed. Although it has a semiaquatic ecology, the feeding behaviour of the postmetamorphic juveniles is similar to that of many aquatic species including *Lithobates catesbeianus,* which often leaves the safety of the water, especially during night-time activity, and feeds mainly on terrestrial insects (Viosca, 1931). Also, the common frog has been previously used in other aquaculture related feeding studies (Miles *et al*., 2004).

Three entire Common frog clutches were collected on February 25, 2018, from three different temporary ponds located in the Faget forest Cluj-Napoca, Cluj county, Transylvania, Romania (46°41'48,57''N 23°32'46,80''E (DMS), Someș-River Basin, elevation 682 m). The egg clutches were kept separately, in three 10 litre aquariums containing dechlorinated tap water. The hatching period lasted for 6 days (03. 27. 2018 - 04. 02. 2018). At Gosner stage 25 (Gosner, 1960) we randomly selected 20 healthy-looking larvae from each clutch (a total of 60 tadpoles). The remaining tadpoles were released in the original ponds.

The selected larvae were raised in 4L opaque containers (9x17x26 cm) at a density on 2,5 individuals / litre, corresponding to low densities in natural populations (Glennemeier and Denver, 2002; Rot-Nikcevic *et al*., 2005). The water temperature was kept constant at 20 ° C ± 1 ° C at a circadian rhythm of 12/12 hours of light / dark. The light was switched on and off at 9am and 9pm

respectively and was provided by four 36W neon tubes. Each container had an independent oxygen supply through electric pumps. Roughly 90% of the water was replaced daily with dechlorinated tap water. The food residues were cleaned permanently, by siphoning.

The diet of the larvae was predominantly of vegetable origin: spirulina (Organic spirulina 500 mg, protein 63.5%, carbohydrates 16.1%, lipids 8.2%. Origin: China), pelleted rabbit feed (Versele Laga Cuni fit pure, protein 15 %, carbohydrates 15% lipids 3% Origins: Hungary) and lettuce (3% protein, 6% carbohydrates, 1% lipid, USDA), supplemented once a week with food of animal origin (lyophilized tubifex, Bio-lio, protein 54 %, lipid 16%, origin: Hungary) (Petranka and Kennedy, 1999).

The metamorphosis period lasted for 7 days (04.26.2018 - 05.02.2018). With the emergence of the forelimbs (ca. Gosner 42), the water quantity was reduced and one end of the container was raised so that the metamorphs can climb out. The metamorphosed frogs were relocated in containers specific to the terrestrial environment.

The postmetamorphic juveniles were held in specially designed opaque plastic containers (27x17x9cm), with textured side walls (Ewert *et al*., 2004), at a density of 4 animals / container. For the substrate, a wash-cloth-like material with high moisture retention capacity was used. A shelter was provided in the form of two branches of artificial plants with leaves (Exoterra forest plant) in order to mimic the natural conditions (Craioveanu *et al*., 2017) and also a terracotta pipe (Ewert *et al*., 2004). In the case of the experimental group, on the opposite side of the shelter we placed a petri dish (Ø 90 mm) as food recipient (Fig. 1).

At the end of the experiment, all surviving animals were released in the original location.

The feeder insect species

Alphitobius piceus (Coleoptera; Tenebrionidae) is a small-sized beetle species (Fig. 2), from the family *Tenebrionidae*, known commonly as the lesser mealworm. In our study, we used the larvae as experimental food.

After hatching, the larva is ca. 1mm long and reaches a maximum of 12 mm before developing into a pupa. The colour is dark yellow to light brown, with variations in intensity, offering a good contrast with both dark and light colour substrates. It feeds on a wide range of plant and animal origin (e.g. dead adults or their own exuviae) foods. The number of instars is variable (9-20) and the pupa stage lasts for 5-30 days, depending on environmental factors. The adults are 5-8mm long, dark brown to glossy black and live ca. 30 days.

The species is a prolific breeder. The female lays about 500 eggs, directly in the substrate. Depending on the temperature and humidity of the environment, the hatching time varies between 5 and 20 days. The sexes are monomorphic.

Lesser mealworms are generally considered cereal pests and are adaptable to a wide range of environmental factors with a very high tolerance threshold. Due to these characteristics, the species is well suited for intensive growth.

In the absence of the species' specific growth protocols and information, and due to the ecological and phylogenetic similarity with the flour beetle (*Tenebrio molitor*), we used as a guide the growth technology developed for the latter.

The growth substrate was prepared according to Klasing *et al*. (2000), with a content of 75% wheat bran (protein 15%, SC Agromar SRL, Romania) and 25% chicken starter feed (Breco, Covasna, Romania). The environmental temperature was 28°C ± 1°C (Fraenkel, 1950) and the environmental humidity approx. 40% with a 24/24 hours lighting consisting of two 36W neon tubes.

The growing containers (60cm L x 30cm l x 20cm h) were half filled with the substrate mixture and seeded with approximately 200 adults. As a water source we used fruits (e.g. a quarter of an apple/week/container). Covering the containers was not necessary because the species does not have the ability to climb vertical surfaces or to fly. The first harvest was made after 60 days. The larvae harvesting method is simple and is done by placing Barber traps, directly in the substrate with pieces of fruit or vegetables used as bait.

Acheta domestiucs (Orthoptera; Gryllidae) is an invasive orthopteran species with an adult body length of 16-21mm and a light brown colour. In this study, the species was used as a control food. The life cycle lasts 8-12 weeks from egg, through 8 larval stages, to adult. The two sexes resemble each-other except for the ovipositor of the female. It is a prolific breeder, lays roughly 50-100 eggs in the substrate with moderate humidity, at a depth of 6-25mm. The hatching period takes about 2 weeks. As adult, the male produces a characteristic stridulating sound.

Farming technology: Adults used for breeding were kept in glass aquariums (60cm L x 30cm l x 35cm h) at a density of ca. 1 cricket / 10 cm^2 (Clifford *et al.*, 1976). To increase the active surface and reduce stress, the bottom of the aquarium was almost completely covered with egg cartons (Nakagaki and Defoliant, 1991). Water was provided by a wash-cloth soaked in water and placed in a petri dish. The ambient temperature was maintained at 28°C ± 1°C, the humidity at about 40% and the lighting was on 24/24 (2 x 36W neon tubes) (Clifford *et al*., 1976).

Food was provided once a day and consisted of a ground mixture (granules of max 1 mm, mesh 150) of granulated feed for rabbits (Versele-Laga, Belgium, Protein 14.0%, Fat 3%, Crude cellulose 20%, Crude ash 7%, Calcium 0.6%, Phosphorus 0.4%), wheat bran (SC Agromar SRL, Romania, Baloteşti, protein 15%) and starter feed for chicken (Breco, Romania, Covasna). In addition to the dry food, we also provided various fresh plants (clover, alfalfa, dandelion) (Nakagaki and Defoliant, 1991; Clifford *et al*., 1976).

For reproduction, we placed egg-laying plastic containers in the adult enclosures (14cm L x 9cm L x 5cm h) filled with high humidity substrate (80-90%). In order to achieve an aerated consistency, the substrate was obtained by mixing potting soil (Agro CS Universal Substrate, Brasov, Romania) with medium-grained sand (Desert Sand, black, Exoterra, USA), in 50:50 ratios. The mere presence of these egg-laying boxes in the breeding colony intensifies the breeding behaviour and triggers ovipositor activity in females. To prevent the cannibalization of eggs by the adults, the boxes were covered with a 2mm wire mesh.

The containers were kept in the breeding colony for 3 days, after which they were replaced with new, unused ones. The seeded containers were moved in plastic boxes (27x17x9cm) equipped with a lid, to maintain humidity. The first larvae hatched after ca. 13 days. Given the high humidity needed for moulting, especially in the early larval stages, the newly hatched larvae were raised in the hatching container until reaching larval stage IV.

We estimated the appropriate size of the prey as the distance between the eyes of the frog. As a consequence, only crickets in larval stage I, II and III were used as feeder for the first two months of the post-metamorphic juveniles' lives.

In order to determine the nutritional value of the two food types used in the experiment, we performed a raw chemical composition analysis (Tab. 1) using Weende's system of analysis.

The chemical analysis was performed at the University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca, Romania, and the feeder insects used in this study were produced in the Vivarium of the Babes-Bolyai Univerity, Cluj-Napoca, Romania.

The study was conducted in accord with the highest humane and ethical principles, according to the ARRIVE guidelines for In Vivo Experiments.

The experimental design

To test the effectiveness of the experimental feeder insect as food for the postmetamorphic juveniles of the common frog, two groups were used: an experimental group, fed with *Alphitobius piceus* larvae and a control group, fed with *Acheta domesticus* larvae. Each group consisted of 20 postmetamorphic

frogs (n=20/group) and was divided into 5 replications (4 animals/replication). We set the size of the 2 groups to n=20 according to previous studies on dietary diversification in frog farms (Miles *et al*., 2004). The experiment lasted for 60 days. At the end of the experimental period, all the surviving animals have reached the necessary maggot eating size.

Measurements and analysis

In order to monitor the evolution of the two groups, we measured and recorded the following variables:

1. Snout to urostyle length, in mm (SUL)

To avoid the excessive handling of animals, a digital imaging technique was used. The retention method was described by Antwis and Browne (Antwis and Browne, 2008, on amphibianark.org), and consists of placing the specimen in a Petri dish and covering it with a lid, in order to obtain a more horizontal position. Also, the thickness of the Petri dish has to be approximately equal to the thickness of the animal. Following this procedure, images were taken of each individual with a Nikon D 3200 digital camera, mounted at a distance of 30 cm above the specimens. The photos were subsequently analysed and measurements were performed using ImageJ open source image processing software (http://imagej.nih.gov/ij).

2. Mass

After the photographs were taken, the animals were dried by placing them briefly on filter paper and weighed with an analytical electronic balance (0.01 g accuracy).

3. Mortality in %

During the 60 days of the experiment (the estimated critical post-metamorphosis time frame), three sets of measurements were performed, on day 0 of the experiment (16.05.2018), on day 30 of the experiment (18.06.2018) and on day 60 (17.07.2018).

The body condition index of the animals was calculated (BCI), according to the residual linear regression model (Băncilă *et al*. 2010), for each treatment (group fed with *Achaeta domesticus* and group fed with *Alphitobius piceus*), and for each measurement session (May 16, June 18 and July 17, 2018). Before performing this analysis, to ensure that the values of SUL and mass meet the criteria for linear regression analysis, for all measurements, we analysed the data as follows:

- graphical analysis using scatter graphs (scatter.smooth function in RStudio)

- graphical analysis using boxplot graphs to identify aberrant values (boxplot function in RStudio)

- graphical analysis using density graphs to highlight the normal distribution of data (density and polygon functions in RStudio)

- calculation of the correlation between SUL and body mass, for each measurement, using Pearson's or Spearman rank correlations, depending on the data distribution (the cor.test function in RStudio).

The preliminary testing of the data was followed by the elaboration of the linear regression model using the function lm (RStudio). The list of the residual values was obtained using the residuals function (RStudio).

The obtained BCI values were tested for normal distribution using the Shapiro-Wilk test and then compared between the two feeding treatments (with *A. domesticus* and *A. piceus*), for each measurement session, using Welch two sample t test.

We also calculated the mortality rate in the two feeding treatments, at each measurement session, and compared them using the Mann-Whitney U-test.

All analyses were performed with Rstudio open source software, version 1.1.463 (2016).

Results

During the three measurement sessions, the average snout to urostyle lengths and body masses were consistently higher for the group fed with crickets (Tab. 2), without the differences between the Body Condition Indexes of the two groups being statistically significant (Tab. 3).

The largest discrepancy between the mean BCI is within the second measurement, with more positive values for the *A. piceus* fed group and more negative for the *A. domesticus* fed group (Tab. 3). The distribution of BCI data (Fig. 3) shows that most values concentrate around 0, indicating a body condition very close to that estimated by the mathematical model for both treatments. There were no differences in BCI between treatments in any of the performed measurements (Tab. 4, Fig. 3).

The mortality percentages of *Rana temporaria* juvenile animals for each replication and each treatment has been summarized in Table 6.

Although the level of animal mortality was consistently higher in the cricket-fed group (Tab. 5), the two groups did not differ significantly from each other (Mann-Whitney test: $p > 0.05$ for each measurement).

Table 1. Raw chemical composition of the experimental (1) and control (2) feeder insect

Species	Dry matter	Crude protein (%) as sampled	Crude protein (%) in dry matter	Crude fat (%) as sampled	Crude fat (%) in dry matter	Crude ash (%) as sampled	Crude ash (%) in dry matter
1. Alphitobius piceus	31.76	18.33	57.74	8.12	25.58	1.40	4.40
2. Acheta domesticus	25.72	16.87	62.79	4.66	18.11	1.69	6.57

Table 2. Mean values, standard deviations and limits of the snout-urosyle length (mm) and body weight (g) during the three measurement sessions

Diet	Ms	N	$\overline{X} \pm s_x$ (Snout-urostyle length - SUL)	$\overline{X} \pm s_x$ (Body weight - BW)	Range (SUL, mm) Mini-mum	Maxi-mum	Range (BW, g) Mini-mum	Maxi-mum
Cricket diet	L1	20	20.51 ±1.49	0.69 ±0.12	18.2	23.1	0.4	0.9
	L2	16	26.19 ±1.97	1.61 ±0.22	21.9	29.4	1.0	2.0
	L3	10	27.69 ±1.40	2.01 ±0.20	25.0	29.8	1.8	2.4
Lesser mealworm diet	L1	20	21.04 ±1.28	0.67 ±0.11	18.1	23.7	0.5	0.9
	L2	18	25.46 ±1.79	1.50 ±0.29	22.0	28.4	1.1	2.1
	L3	12	26.99 ±1.57	2.09 ±0.37	24.0	29.1	1.7	2.8

Ms=measurement session; L1=first measurement; L2=second measurement; L3=third measurement; N=group size; $\overline{X}$=mean; s_x=standard deviation; Range=extreme values of the measurements;

Table 3. Mean values and limits for the body condition index of the animals fed with the two species of experimental food (*A. domescus* and *A. piceus*)

Measurement session	Experimental food	$\bar{X}\pm$	s_x	Range	
				minimum	maximum
L1	*A. domesticus*	$-3.4 * 10^{-07}$	±0.8786	-1.67413	1.723816
L1	*A. piceus*	$-1* 10^{-09}$	±0.8179	-1.84637	1.234487
L2	*A. domesticus*	$-1.3 * 10^{-09}$	±1.4340	-2.00525	3.346637
L2	*A. piceus*	$1.11 * 10^{-09}$	±1.1797	-2.39603	1.556069
L3	*A. domesticus*	$-1.1 * 10^{-09}$	±0.7361	-1.20132	1.597073
L3	*A. piceus*	$-1.4 * 10^{-09}$	±1.2179	-2.46773	1.450089

L1=first measirement; L2=second measurement; L3=third measurement; $\bar{X}$=mean; s_x=standard deviation; Range=extreme values of the measurements

Table 4. Results of comparisons between body condition indices of R. temporaria animals fed with the two feeder insect species (Welch two sample t-test), in the three measurement sessions

Measurement	Value of t	Degrees of freedom	Value of p
16 May (L1)	$-1.272 *10^{-06}$	37.807	1
18 June (L2)	$-5.204 *10^{-09}$	29.164	1
17 July (L3)	$7.787 *10^{-10}$	20.969	1

Table 5. Mean values and range of the mortality (%) of the animals for the two experimental groups for each replication and for the three measurement sessions

***Acheta* sp.**	**N**	$\bar{X} \pm s_x$		**Range**	
				minimum	**maximum**
Measurement 1	5	0		0	0
Measurement 2	5	20±	20.917	0	50
Measurement 3	5	55±	11.18	50	75
***Alphitobius* sp.**					
Measurement 1	5	0		0	0
Measurement 2	5	10±	13.693	0	25
Measurement 3	5	30±	20.916	0	50

Figure 1. Enclosure for postmetamorphic frogs.

Figure 2. *Alphitobius piceus*, larvae and imago.

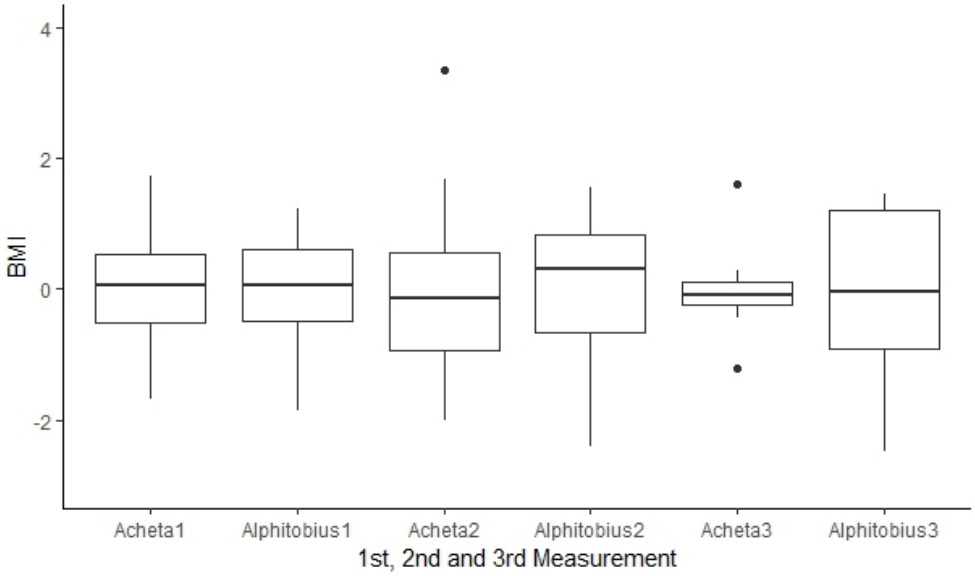

Figure 3. Values of the body condition indices (BMI) of the animals in the two different treatments (animals fed with *Acheta domesticus* and animals fed with *Alphitobius piceus*) during the three measurement sessions (1st-May 16, 2nd-June 18 and 3rd-July 17). Plots represent median (line inside boxes), 25–75 percent quartiles (boxes) and minimal and maximal values. Outliers are represented with a dot.

Discussion

Providing the right type of food and also a good management of the associated costs is a pivotal point in frog farming and represents important indicators in anticipating the success or failure of a commercial enterprise.

Although frogs are highly specialized predators, the search for novel feeder species or inert peleted food items will be a constant preoccupation of the industry and the related scientific comunity (Lima *et al*., 1986; Rodriguez-Serna *et al*., 1996; Braga *et al*., 1998; Castro *et al.,* 2001).

The aim of this study was to identify an alternative, low cost, food item for the juvenile postmetamorphic frogs in the most vulnerable period of their life cycle. We also tested if this type of food has an appropriate quality.

Considering the trophic needs of the frogs in early postmetamorphic development, the two compared diets showed a high similarity, in terms of crude protein and crude ash (Tab. 1). This similarity was indeed reflected in no significant differences between the control and the experimental group from the perspective of the body condition index (Tab. 4).

From a behavioural point of view, the two species could have elicited different feeding responses, considering the lower speed and mobility of the experimental insect. However, in our study, the experimental food was easily accepted and the specific" worm like" shape produced a strong feeding response. The results are in line with earlier neuro-ethological (Ewert, 1980) and neuro-physiological (Beauquin and Gaillard,1998) studies that indicated a strong preference of amphibians towards horizontal, slow moving, prey-like objects.

Regarding the mortality rate, although statistically insignificant, there was an important difference between the two groups (55% group fed *A. domesticus* versus 30% group fed *A. piceus*; Tab. 5, measurement no. 3). We cannot exactly identify the source of this difference, but we suppose that it comes from the method of presenting the two types of feeder insect species. Cricket larvae were released freely into the rearing containers while the lesser mealworms were offered in a Petri dish. Consequently, the cricket-fed animals had to actively hunt for prey, with probably less success, while those fed on lesser mealworms, once accustomed with the location of the food, were able to feed without significant effort.

Conclusions

An overall conclusion of this study is that the experimental feeder insect produces similar level of nutritional performances as the control feeder insect, in laboratory conditions.

In the real-world conditions of a frog farm, we believe that *Alphitobius piceus* has key characteristics to enhance productivity at a potentially lower cost and improve economic profitability.

As a delivery method, we recommend placing the larvae in shallow feeding trays, in the close proximity of the shoreline. As a result of this layout, the juvenile frogs will have good access to food, with most of the feeding occurring during night time activity.

If used for a longer period, there is a reasonable expectation that the experimental insect would be better accepted in time, considering that exposure to a particular prey strengthens the feeding behaviour response to that prey in amphibians (Jaeger and Barnard, 1981). Also, according to stomach content analysis, many frog species prey largely on adult beetles in the wild (Korschgen and Moyle, 1955; Stojanova and Mollov, 2008). The adult of *Alphitobius piceus* has a number of characteristics that could simplify its large scale production (e.g. the adult does not fly, does not have the ability to climb vertical surfaces), therefore, although it has not been the subject of this study, we consider that the adult stage also represents a good potential food item in frog farming.

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