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Are mixed diets beneficial for the welfare of captive axolotls (*Ambystoma mexicanum*)? Effects of feeding regimes on growth and behavior

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Abstract

Good nutritional husbandry is crucial to maintain high welfare standards in captive animals. Both direct effects of diet on growth, development, and maintenance, and indirect effects of feeding regimes on behavior may be important. Despite this, many questions remain as to how we should best feed many of the species that are commonly kept in captivity. There is a great deal of speculation amongst animal keepers as to issues such as whether a mixed diet is better than an invariant one, but little research is available to inform this question. In this study, we investigate the impact of mixed versus invariant diets on growth and behavior in the axolotl (*Ambystoma mexicanum*), an aquatic amphibian of severe conservation concern that is frequently maintained in captive collections. We then use our results to provide advice on feeding management in the context of improved welfare. We maintained juvenile axolotls under one of three 'diets' (feeding regimes): bloodworm (invariant), *Daphnia* (invariant), and alternating these two prey items between feeds (mixed). Morphological and behavioral data were collected over a period of 15 weeks and analyzed using generalized linear mixed models to determine whether our feeding treatments influenced growth and behavior. We find that axolotls grew fastest on our bloodworm diet and slowest on our *Daphnia* diet, with a mixed feeding regime leading to intermediate growth rates. Diet treatment did not significantly influence our measured behaviors, but feeding and locomotion events were more frequent (and resting less frequent) on feeding days than non-feeding days. These data suggest that providing a mixed diet is not necessarily beneficial to either growth or welfare of captive animals. In the case of axolotls, an invariant diet of bloodworm should increase growth rates but the diet (mixed versus invariant) does not influence behavior. Overall, our results suggest that mixed diets in themselves may not be beneficial to the growth or welfare of axolotls as compared to a high-quality invariant diet.

Keywords: Development; Nutrition; Folklore husbandry; Aquatic amphibian; Environmental enrichment; Activity

Introduction

Studies of diets and feeding regimes are important to promote good nutrition in captive animals by allowing an evidence-based husbandry approach. Adequate nutrition is necessary for optimal growth, maintenance, health and reproduction (Oftedal and Allen, 1996); therefore failure to provide suitable diets can negatively impact captive breeding programs and animal welfare. For instance, many common veterinary conditions including metabolic bone diseases, obesity, anorexia, nutrient deficiencies and toxicities, and some infectious diseases are a direct result of poor dietary management (Donoghue, 2006; Rosenthal and Mader, 2006). Furthermore, indirect benefits of good nutritional resources are also evident. For instance, Venesky et al. (2012) found that leopard frog tadpoles (*Lithobates sphenocephalus*) fed a high-protein diet had greater immune function and resistance to the cosmopolitan epizootic chytrid fungus (*Batrachochytrium dendrobatidis*) when compared to tadpoles fed a low-protein diet. Therefore, nutrition is a vital consideration for animal husbandry if we are to maintain high welfare conditions (Hadfield et. al., 2006).

Evidence-based husbandry is an important goal, but there remains limited research available upon which such approaches can be built. While this applies to captive animals in general, ecological and husbandry-related research suffers from a taxonomic bias towards mammals (Bonnet et al., 2002; Anderson et al., 2008; Arbuckle, 2009; Hosey et al., 2009), and amphibians are particularly poorly represented in nutritional studies (Arbuckle, 2009). As such, if we are to implement evidence-based husbandry regimes to improve welfare of captive amphibians (and other animals) we must first generate a good research platform from which to start. Indeed, many non-evidence-based (or 'folklore') husbandry practices and claims concerning exotic animals have been found to be poorly justified upon academic scrutiny (e.g. Arbuckle, 2010).

The animal care literature is replete with claims that mixed diets are better than invariant, single prey-species, diets for carnivorous species (e.g. Greene et al., 1997; Preece, 1998; Barrie, 1999; Calvert, 2004; Barten, 2006; Diaz-Figueroa, 2008). However, few studies have investigated whether mixed diets provide advantages for the growth, development or behavior of captive animals, and so assertions of increased welfare are generally examples of folklore husbandry (Arbuckle, 2013). Mehrparvar et al. (2013) investigated whether single or multiple aphid species fed to insect predators improved the development or survival of the predators, and in fact found that mixed diets were inferior to a good single prey species. Borg and Toft (2000) used a gradient of mixed diets (aphids and grasshoppers) from 0% to 45% aphids plus a 'free choice' condition to feed grey partridge chicks. Their study was designed to test optimal foraging predictions with regard to diet choice, but the data suggested that a small amount of aphids in the diet was much better than a high proportion of aphids and slightly better than no aphids (an invariant diet of grasshoppers) in terms of growth. This suggests that there may be a slight benefit to mixed diets for some species, although Borg and Toft (2000) did not explicitly test this question. Given the conflicting evidence between studies on different animal groups, it is notable that no research is yet available on many groups commonly maintained in captivity, such as amphibians.

Axolotls (*Ambystoma mexicanum*) are neotenic salamanders kept in large numbers in captivity, including in the pet trade, zoos, aquariums, museums, and in laboratories. They are listed as critically endangered in the International Union for Conservation of Nature Red List of Threatened Species since 2006 as they occupy an area of approximately $10km^2$ or less and are threatened by habitat degradation (IUCN, 2008). Previous conservation efforts have ranged from habitat restoration to reintroductions, and axolotls have been used as a flagship species due to their status as a charismatic species that may engage members of the public to support their conservation (Simberloff, 1998; Caro and O'Doherty, 1999). However, populations have continued to decline to the extent that they may be extinct in the wild and the species may be heavily reliant on the captive population to ensure its survival. Amphibians have suffered global population declines (Stuart et al., 2004; Beebee and Griffiths, 2005) and managed captive breeding programs have been recognized as an important conservation tool (Griffiths and Pavajeau, 2008). Therefore, research aimed at improving husbandry for axolotls and other amphibians is important both for the welfare of the vast number of individuals in captivity and for the conservation of threatened species. Nutrition is an important facet of husbandry for these aims (Oftedal and Allen, 1996).

We fed axolotls on diets consisting of either one of two prey species (bloodworm or *Daphnia*) or a mixed diet consisting of both prey types to investigate whether a mixed diet was beneficial. We measured both morphology and behavior to assess the effect of diet on growth, development, and welfare (using behavior as a proxy). We predicted that, if mixed diets are beneficial, axolotls in this experimental treatment would grow faster, reach a larger size, and exhibit more activity such as locomotion than axolotls fed either invariant diet.

Materials and Methods

Study animals and general husbandry

We acquired 24 axolotls from a local breeder. All individuals were siblings and hatched in April 2013. Axolotls were randomly (using a random number generator) assigned to one of six separate and identical tanks, ensuring only that each tank was assigned four individuals. Dechlorinated water, a filter, shelters for hiding (in the form of a perforated building brick), and an aerating stone were provided in each tank. Cleaning was carried out once per week, including an approximately one-third water change. Axolotls were housed in a laboratory setting at Liverpool John Moores University.

All axolotls were left to acclimate for one week before the experiments, during which time they were fed on a mixed diet of two frozen/thawed prey species: bloodworm and *Daphnia*. These two prey species are commonly used for captive axolotls and therefore maintain the realism and applicability of our experiments to a practical setting. Thereafter, for the 15 week duration of the experiment, two tanks each were assigned to one of three separate diets: two invariant diets (bloodworm only or *Daphnia* only) and a mixed diet (alternating between bloodworm and *Daphnia* on subsequent feeding days). All axolotls were fed three times per week (Monday, Wednesday, and Friday). Total quantity of food was increased over the course of the experiment to account for increasing size of the animals (initially 1.5g, increasing by 0.25g every two weeks until a maximum of 2.5g per tank), but food quantities were identical across diet treatments.

We used digital photographs of natural tail markings to identify individual axolotls, a common, non-invasive, and reliable method for amphibians (Caorsi et al., 2012). We first verified that we could accurately identify each individual from these photographs and then, in order to ensure that reliability did not decline with growth, they were regularly updated during the course of our experiment.

Morphological data

Body mass (g) was measured once per week by placing each axolotl in a petri dish and using a laboratory balance with an accuracy of 0.01g. Each measurement was taken three times and the mean was recorded as our measure of body mass.

Snout-vent length (cm), torso width (cm) and head width (cm) were recorded each week using digital photographs taken from above. A tripod was used to standardize the distance and angle between the camera and axolotl. These photographs included a sheet of graph paper to enable us to calibrate the scale and our three measures were calculated using ImageJ version 1.41 (Rasband, 1997-2014).

Behavioral data

Behavioral observations were made using instantaneous sampling (*sensu* Altmann, 1974) of each individual at 10 second intervals for one minute (including time 0, giving 7 observations per individual per sampling period). Sampling of every individual was conducted on two days each week: one on a feeding day ('food present'), and one on a non-feeding day ('food absent'). On feeding days, observations were made five-ten minutes after introducing food to the tank. Prior to the start of the experiment pilot observations were made to assess which behaviors were performed by the axolotls, and these were used to create an ethogram (Table 1). Of these behaviors (feeding, locomotion, resting, spitting, and time out), spitting was too rare to allow meaningful analysis and time out was of limited value to interpretation. Therefore analyses of behavioral data were conducted on the other behaviors separately as the proportion of samples in which they were recorded in each observation period. Because the axolotls could not be observed during time out behavior (by definition, see Table 1), these were excluded such that the proportions were calculated based on samples when the individual was visible. We should also clarify that despite our terminology of 'food present' versus 'food absent', feeding was possible even on non-feeding days as some food was typically left over from the previous feeding day. Nevertheless, there was usually little food left over and this was often partially decomposed, so although possible, feeding opportunities were far more limited on non-feeding compared to feeding days.

Data analysis

In order to control for individual differences in growth and behavior, all analyses were conducted using generalized linear mixed models (GLMMs) performed in the lme4 package version 1.0-4 (Bates et al., 2013) in R version 3.0.1 (R Core Team, 2013). Model fitting started with a 'full model', containing all explanatory variables and their two-way interactions. The final, or 'best', model was selected using stepwise model selection wherein the simpler model at each stage was accepted if it did not provide a significantly poorer fit to the data based on analysis of deviance (a standard means of comparing nested models, see Thomas et al., 2013).

Morphological variables were modelled with a Gaussian error structure, and residuals of all models were visualized to check for normality. GLMMs were fit for each response variable (body mass, snout-vent length, torso width and head width) using diet treatment, time (as week of the experiment), and their interaction as explanatory variables and with individual as a random effect in the full model.

Behavioral variables were converted to proportions of total events (excluding time out) per sampling period using the cbind function in R and then modelled with a binomial error structure. GLMMs were fit for each response variable (proportion of samples feeding, locomotion, and resting) using 'food present/absent', diet treatment, time (as week of the experiment), and their two-way interactions as explanatory variables and individual as a random effect in the full model.

Results

All of our morphological variables showed the same structure in our best models (Table 2). There was a significant interaction between growth (body size as a function of time) and diet, such that axolotls fed an invariant bloodworm diet grew significantly faster than those on a mixed diet, which in turn grew significantly faster than those fed an invariant *Daphnia* diet (Table 2; Figure 1). The effect of diet treatment on growth was slightly less pronounced in torso width compared to body mass, snout-vent length, and head width (Figure 1), but significant in all cases (Table 2).

In contrast, only the 'presence of food' (feeding versus non-feeding days) influenced our behavior traits according to our best models (Table 3). During feeding days, axolotls exhibited more feeding and locomotion behavior and less resting behavior compared to non-feeding days (Figure 2). The particular diet treatment had no significant effect on behavior and we did not find that behavior changed over the course of our experiment.

Discussion

This study aimed to assess whether mixed diets are inherently better than invariant diets for the welfare of captive animals, as is often assumed. We looked for the influence of feeding regime on growth (in four morphological traits: body mass, snout-vent length, torso width, and head width) and behavior in axolotls. We found that bloodworm-only diets produced higher growth rates than a mixed diet (or a *Daphnia*-only diet), and that these three treatments had no influence on the behaviors recorded herein. Because increased activity and other such behavior is frequently used as a proxy for welfare and successful enrichment (Newberry, 1995; Hosey et al., 2009), we suggest that mixed diets are not necessarily better for the growth or welfare of captive axolotls.

The higher growth rates in bloodworm-fed axolotls compared to those fed mixed or *Daphnia* diets is likely due to the higher protein and fat content of bloodworm versus *Daphnia* (5% versus 2.4% protein, 1% versus 0.7% fat). Therefore the additional nutritional resources available from bloodworm confer the ability to grow quicker than when fed *Daphnia*, or in a mixed diet where the nutrient content of bloodworm is 'diluted' with that of *Daphnia*. Since the two prey species in the mixed diet differ in nutrient composition, it is possible that the impacts on growth in this study are a result of lower nutrition and not that the diet was mixed *per se*. However, in practice, a mixed diet rarely consists of nutritionally-matched prey, and so a claim that mixed diets are better must stand up to differences in nutritional quality between prey items. Since the prey items we chose are commonly used in axolotl husbandry, our experiments assess such claims in a realistic way that is applicable to actual captive care regimes. Nevertheless, we acknowledge that a similar experiment with prey items matched for nutritional value would provide further insights into the perceived benefit of mixed diets.

In contrast to our results, Aquilino et. al. (2012) found that the turban snail (*Chlorostoma funebralis*) and the lined shore crab (*Pachygrapsus crassipes*) displayed a higher growth when fed a variety of algal species compared to single algal species. However, it is possible that differences in nutrient composition amongst plant or fungal species are greater than that amongst animal species due to differential micronutrient uptake of primary producers. If this is the case then we might expect herbivores to react differently to mixed diets than carnivores. Indeed, amongst captive exotic animals, many carnivores are typically considered to do well on a single prey item, whereas herbivores may be more likely to have problems such as refusal to feed on such diets (Funk, 2006; Arbuckle, 2010). In any case, axolotls appear to have higher growth rates when fed on a nutritionally-rich (rather than a varied) diet. Since feeding behaviors did not show a decrease with time (Table 3), we also present evidence that axolotls do not refuse to feed when fed an invariant diet, at least over a 15 week period, arguing against the type of issues noted in some other species (Funk, 2006).

Although our finding of increased activity (both feeding and locomotion) and decreased resting when food is present is unsurprising, we failed to find any effect of diet treatment on behavior. We initially predicted that a mixed diet may be enriching and provide benefits to welfare as manifest through an increased activity, either via motivation effects of a varied diet or by requiring greater movement to capture different types of prey. This prediction was in line with the common folklore husbandry claim that varied diet are in some way ‘better’ than invariant diets. Our data provide no evidence to support this and suggest that, similar to Mehrparvar et al.’s (2013) findings in aphid predators, mixed diets are not necessarily a better choice when feeding animals.

We urge caution when using our results because we only investigated the effects of mixed diets on behavior and morphology. It is possible that dietary factors influence physiological function such as immune response (Kelly & Tawes, 2013), and mixed diets could have benefits here that we were unable to measure in our study. Specifically, Kelly & Tawes (2013) found that female crickets fed a lower quality diet actually had better immune function, presumably due to preferential investment of resources, although male crickets showed no such effect. Therefore under this scenario the lower quality *Daphnia* diet may improve immune function and a mixed diet could provide a compromise between a better immune response and more nutritional resources in axolotls. However, this may not be generalizable since Venesky et al. (2012) found the opposite result in an amphibian – that higher quality diets conferred higher resistance to the pathogenic chytrid fungus. Consequently, the influence of a mixed diet on aspects of health and welfare other than those considered here remain unknown in axolotls, although our study still provides evidence from a morphological/developmental and behavioral perspective.

We would also like to stress that we are not recommending an overly general interpretation of our results to say that invariant diets are beneficial for captive animals as a whole. Different species are likely to respond in different ways to diet variability and the nutrient content of captive diets is also likely to vary between classes of food items (e.g. herbivorous versus carnivorous diets, vertebrate versus invertebrate feeders). Nevertheless, we show that mixed diets have no descernable impact on behavior of axolotls and result in a slower growth rate than a bloodworm-only diet. For this common laboratory and pet species, and perhaps other amphibians or aquatic carnivores, is seems that an invariant but good quality diet is a better option. At the very least, our results highlight that the dogma of mixed diets being best is not universally true.

This paper contributes to the growing literature addressing examples of folklore husbandry (e.g. Schwitzer et al., 2008; Arbuckle, 2009, 2010; Ferguson et al., 2010; Rosier & Langkilde, 2011). Testing such claims is an important step towards improving our husbandry regimes and potentially allows us to achieve better success in captive breeding, increase welfare standards, and perhaps reduce time and financial costs (Arbuckle, 2013). Furthermore, in the case of the axolotl, which is not only commonly held in captivity but also threatened in the wild, amassing evidence to inform husbandry can improve conservation programmes. This is particularly important considering the recognized importance of *ex situ* approaches to amphibian conservation (Griffiths and Pavajeau, 2008), for which good quality husbandry conditions are vital to the success of any strategy.

Conclusions

We found no advantage to a mixed diet over a high quality single-prey-species diet for the growth or behavior of axolotls. Diet variability had no influence on behavior and, in the case of growth, bloodworm-only diets performed significantly better than a mixed diet. We suggest that for this species, and possibly other amphibians or aquatic carnivores, a good-quality invariant diet is a better strategy than a mixed diet. More generally, this paper adds to the growing literature aimed at providing a platform for evidence-based husbandry (*sensu* Arbuckle, 2013). Continued research in this vein is required if we are to promote good captive management practices, improve welfare standards, and inform conservation efforts for amphibians and other species.

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Ethical Statement

The work described in this article was approved by Liverpool John Moores University. Furthermore, the procedures were non-invasive, experimental conditions were non-stressful, and the husbandry regime was designed to incorporate accepted standards of welfare for axolotls. The work was carried out in a manner consistent with the Association for the Study of Animal Behaviour's 'guidelines for the treatment of animals in behavioural research and teaching'.

Conflict of Interest Statement

None of the authors have any conflicts of interests that could be deemed to influence the objectivity of this work.

Author Contributions

The idea for the paper was conceived by DS, HJN, and KA. The experiments were designed by DS, HJN, and KA. The experiments were performed by DS. The data were analyzed by DS and KA. The paper was written by DS, KA and HJN.

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Table 1 - Ethogram for behaviors recorded in this study

Behavior	**Description**
Feeding	Ingestion of foodstuffs
Locomotion	Movement without other accompanying behaviors
Resting	No movement or display of other behaviors
Spitting	The forceful expulsion of items (e.g. food) from the mouth
Time out	Out of view of observer

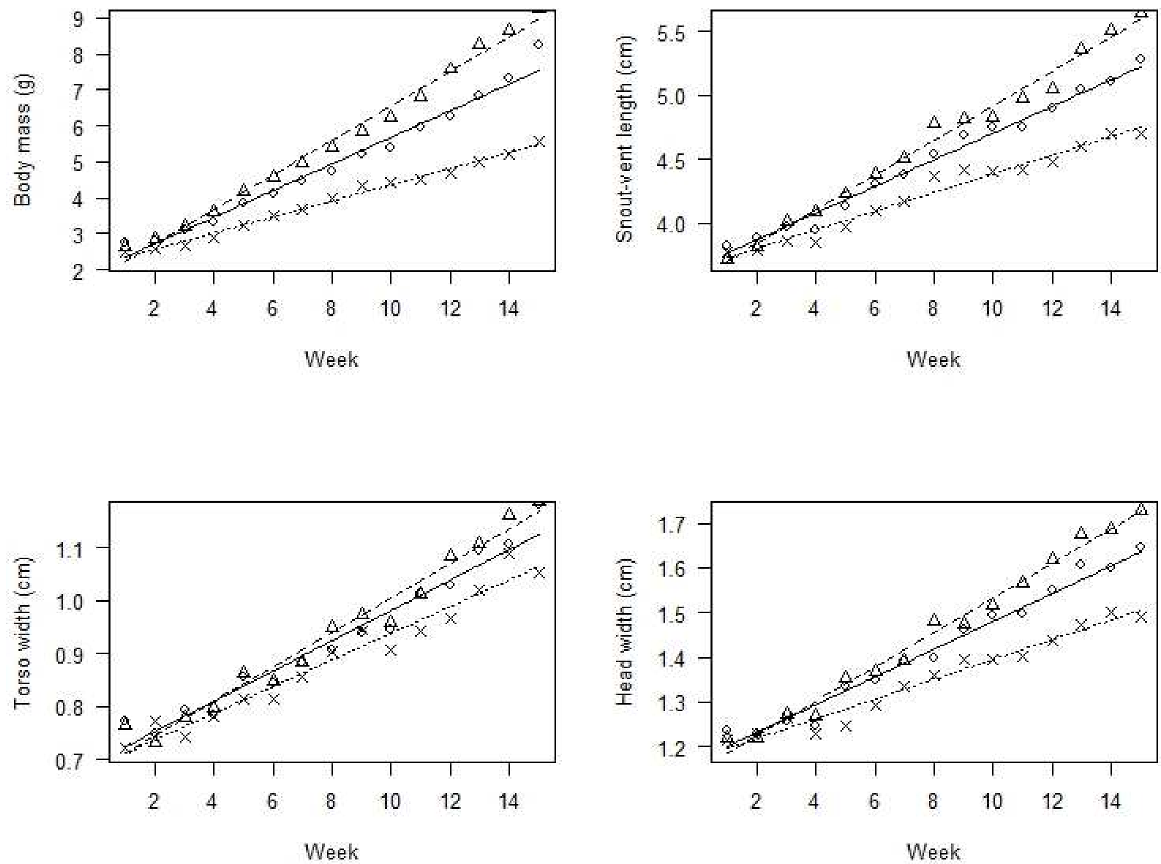
Table 2 – Results from the best model for each morphological variable. All models are GLMMs controlling for individual as a random effect. For all morphological variables the best model includes a significant interaction between diet and time, indicating that diet influenced growth over the course of the experiment. Effects of diet treatments were estimated as contrasts to the mixed diet. N=359.

Response variable	**Explanatory variable(s)**	**β ± SE**	**t**	**P**
Body mass	Constant	2.126 ± 0.510	4.167	<0.001
	Bloodworm	-0.513 ± 0.567	-0.905	0.36
	Daphnia	0.006 ± 0.794	0.008	0.99
	Time	0.369 ± 0.014	24.803	<0.001
	Bloodworm x time	0.111 ± 0.021	5.284	<0.001
	Daphnia x time	-0.145 ± 0.021	-6.952	<0.001
Snout-vent length	Constant	3.576 ± 0.151	23.551	<0.001
	Bloodworm	0.077 ± 0.151	0.511	0.60
	Daphnia	0.078 ± 0.241	0.325	0.74
	Time	0.104 ± 0.003	28.576	<0.001
	Bloodworm x time	0.030 ± 0.005	5.792	<0.001
	Daphnia x time	-0.032 ± 0.005	-6.119	<0.001
Torso width	Constant	0.701 ± 0.038	18.091	<0.001
	Bloodworm	-0.03 ± 0.045	-0.665	0.50
	Daphnia	-0.014 ± 0.059	-0.250	0.80
	Time	0.028 ± 0.001	22.923	<0.001
	Bloodworm x time	0.004 ± 0.001	2.314	0.02
	Daphnia x time	-0.003 ± 0.001	-1.955	0.05
Head width	Constant	1.165 ± 0.042	27.623	<0.001
	Bloodworm	-0.012 ± 0.045	-0.269	0.78
	Daphnia	0.009 ± 0.065	0.145	0.88
	Time	0.031 ± 0.001	26.287	<0.001
	Bloodworm x time	0.007 ± 0.001	4.473	<0.001
	Daphnia x time	-0.008 ± 0.001	-5.387	<0.001

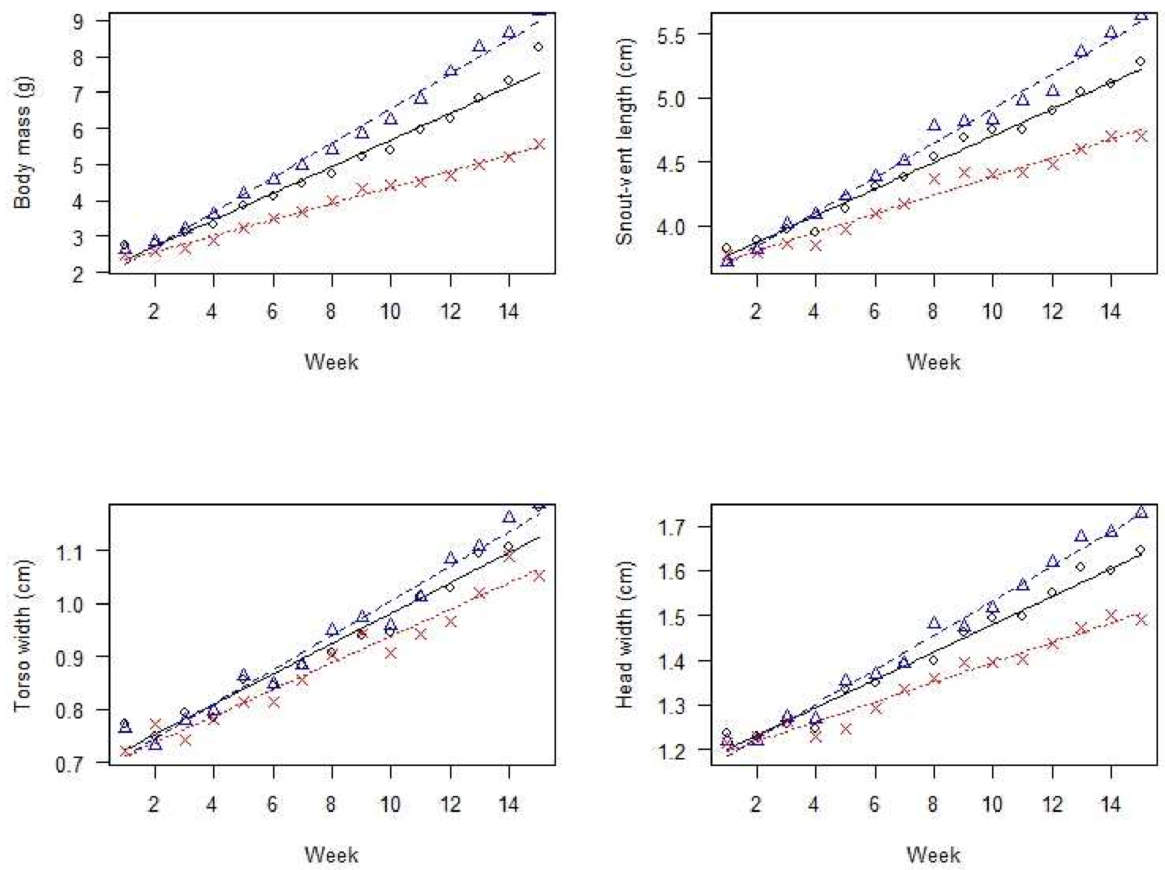
Table 3 - Results from the best model for each behavior of interest. All models are GLMMs controlling for individual as a random effect. All behaviors were influenced only by the presence of food. There was no significant effect of diet treatment nor was there a change in any behavior over the course of the experiment. N=718.

Response variable	Explanatory variable(s)	β ± SE	z	P
Feeding	Constant	-5.431 ± 0.302	-17.98	<0.001
	Food present	4.300 ± 0.304	14.11	<0.001
Locomotion	Constant	-2.422 ± 0.070	-34.66	<0.001
	Food present	1.300 ± 0.081	16.12	<0.001
Resting	Constant	-0.195 ± 0.030	-6.58	<0.001
	Food present	-1.315 ± 0.056	-23.69	<0.001

Figure 1 – Growth (increase in size over the duration of the experiment) varies with diet in all four measures of size used herein. Lines are the predictions from our GLMMs, and points are mean values for each diet treatment in each week. Dashed lines and triangles represent a bloodworm diet, solid lines and circles represent a mixed diet, solid lines and crosses represent a *Daphnia* diet. Axolotls fed an invariant bloodworm diet grew fastest, followed by those fed a mixed diet, and *Daphnia*-fed individuals grew slowest.

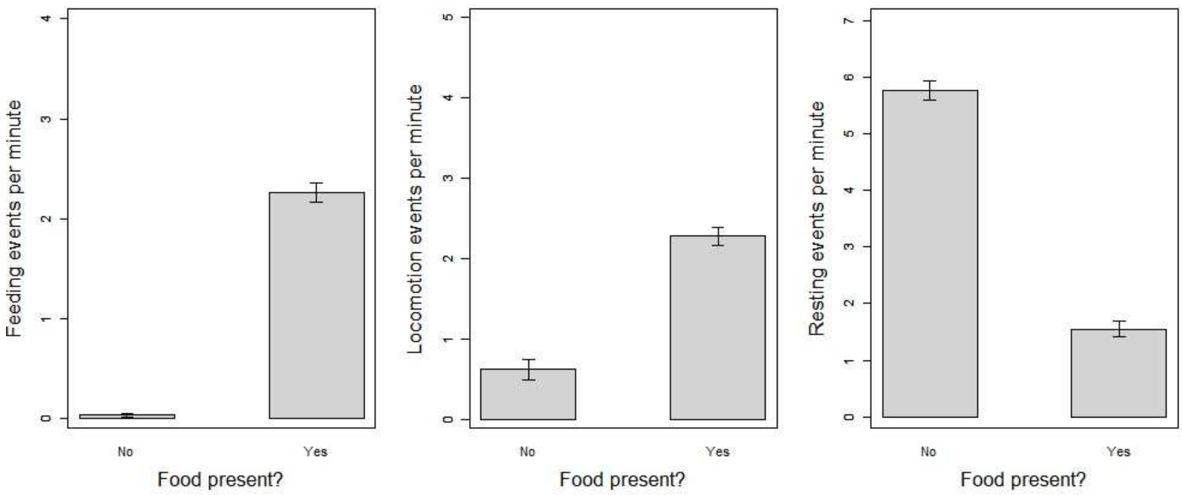


GREYSCALE VERSION FOR PRINT PUBLICATION (FIGURE 1)



COLOR VERSION FOR ONLINE PUBLICATION (FIGURE 1)

Figure 2 – Behavior was only influenced by the presence of food, not diet treatment. Feeding and locomotion behaviors increased and resting decreased on feeding days compared to non-feeding days. Error bars are 95% confidence intervals. Behavioral events per minute are based on scan samples taken at 10 second intervals over one minute per individual (i.e. 7 samples per minute).



- Groups of axolotls were fed bloodworm, *Daphnia*, or a mixed diet.
- Morphometric and behavioural measurements over time were recorded.
- Axolotls grew best on an invariant bloodworm diet.
- Bloodworm-fed animals were more active than others, though a mixed diet may temporarily increase activity.
- Despite common perceptions, mixed diets do not necessarily provide improved welfare compared to invariant diets.