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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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regimes on growth and behavior

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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.

42

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

45 **Introduction**

49 1996); therefore failure to provide suitable diets can negatively impact captive breeding
50 programs and animal welfare. For instance, many common veterinary conditions including
51 metabolic bone diseases, obesity, anorexia, nutrient deficiencies and toxicities, and some
52 infectious diseases are a direct result of poor dietary management (Donoghue, 2006;
53 Rosenthal and Mader, 2006). Furthermore, indirect benefits of good nutritional resources are
54 also evident. For instance, Venesky et al. (2012) found that leopard frog tadpoles (*Lithobates*
55 *sphenocephalus*) fed a high-protein diet had greater immune function and resistance to the
56 cosmopolitan epizootic chytrid fungus (*Batrachochytrium dendrobatidis*) when compared to
57 tadpoles fed a low-protein diet. Therefore, nutrition is a vital consideration for animal
58 husbandry if we are to maintain high welfare conditions (Hadfield et. al., 2006).

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

69 The animal care literature is replete with claims that mixed diets are better than
70 invariant, single prey-species, diets for carnivorous species (e.g. Greene et al., 1997; Preece,
71 1998; Barrie, 1999; Calvert, 2004; Barten, 2006; Diaz-Figueroa, 2008). However, few studies
72 have investigated whether mixed diets provide advantages for the growth, development or

76 predators, and in fact found that mixed diets were inferior to a good single prey species.
77 Borg and Toft (2000) used a gradient of mixed diets (aphids and grasshoppers) from 0% to
78 45% aphids plus a 'free choice' condition to feed grey partridge chicks. Their study was
79 designed to test optimal foraging predictions with regard to diet choice, but the data
80 suggested that a small amount of aphids in the diet was much better than a high proportion
81 of aphids and slightly better than no aphids (an invariant diet of grasshoppers) in terms of
82 growth. This suggests that there may be a slight benefit to mixed diets for some species,
83 although Borg and Toft (2000) did not explicitly test this question. Given the conflicting
84 evidence between studies on different animal groups, it is notable that no research is yet
85 available on many groups commonly maintained in captivity, such as amphibians.

86 Axolotls (*Ambystoma mexicanum*) are neotenic salamanders kept in large numbers
87 in captivity, including in the pet trade, zoos, aquariums, museums, and in laboratories. They
88 are listed as critically endangered in the International Union for Conservation of Nature Red
89 List of Threatened Species since 2006 as they occupy an area of approximately 10km² or
90 less and are threatened by habitat degradation (IUCN, 2008). Previous conservation efforts
91 have ranged from habitat restoration to reintroductions, and axolotls have been used as a
92 flagship species due to their status as a charismatic species that may engage members of
93 the public to support their conservation (Simberloff, 1998; Caro and O'Doherty, 1999).
94 However, populations have continued to decline to the extent that they may be extinct in the
95 wild and the species may be heavily reliant on the captive population to ensure its survival.
96 Amphibians have suffered global population declines (Stuart et al., 2004; Beebee and
97 Griffiths, 2005) and managed captive breeding programs have been recognized as an
98 important conservation tool (Griffiths and Pavajeau, 2008). Therefore, research aimed at
99 improving husbandry for axolotls and other amphibians is important both for the welfare of

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

108

109 **Materials and Methods**

110 *Study animals and general husbandry*

111

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

119 All axolotls were left to acclimate for one week before the experiments, during which
120 time they were fed on a mixed diet of two frozen/thawed prey species: bloodworm and
121 *Daphnia*. These two prey species are commonly used for captive axolotls and therefore
122 maintain the realism and applicability of our experiments to a practical setting. Thereafter, for
123 the 15 week duration of the experiment, two tanks each were assigned to one of three
124 separate diets: two invariant diets (bloodworm only or *Daphnia* only) and a mixed diet
125 (alternating between bloodworm and *Daphnia* on subsequent feeding days). All axolotls

129 food quantities were identical across diet treatments.

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

135

136 *Morphological data*

137

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

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

146

147 *Behavioral data*

148

149 Behavioral observations were made using instantaneous sampling (*sensu* Altmann, 1974) of
150 each individual at 10 second intervals for one minute (including time 0, giving 7 observations

154 the tank. Prior to the start of the experiment pilot observations were made to assess which
155 behaviors were performed by the axolotls, and these were used to create an ethogram
156 (Table 1). Of these behaviors (feeding, locomotion, resting, spitting, and time out), spitting
157 was too rare to allow meaningful analysis and time out was of limited value to interpretation.
158 Therefore analyses of behavioral data were conducted on the other behaviors separately as
159 the proportion of samples in which they were recorded in each observation period. Because
160 the axolotls could not be observed during time out behavior (by definition, see Table 1),
161 these were excluded such that the proportions were calculated based on samples when the
162 individual was visible. We should also clarify that despite our terminology of ‘food present’
163 versus ‘food absent’, feeding was possible even on non-feeding days as some food was
164 typically left over from the previous feeding day. Nevertheless, there was usually little food
165 left over and this was often partially decomposed, so although possible, feeding
166 opportunities were far more limited on non-feeding compared to feeding days.

167

168 *Data analysis*

169

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

181 time (as week of the experiment), and their interaction as explanatory variables and with
182 individual as a random effect in the full model.

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

189

190 **Results**

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

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

203

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

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

228 In contrast to our results, Aquilino et. al. (2012) found that the turban snail
229 (*Chlorostoma funebris*) and the lined shore crab (*Pachygrapsus crassipes*) displayed a
230 higher growth when fed a variety of algal species compared to single algal species. However,

234 diets than carnivores. Indeed, amongst captive exotic animals, many carnivores are typically
235 considered to do well on a single prey item, whereas herbivores may be more likely to have
236 problems such as refusal to feed on such diets (Funk, 2006; Arbuckle, 2010). In any case,
237 axolotls appear to have higher growth rates when fed on a nutritionally-rich (rather than a
238 varied) diet. Since feeding behaviors did not show a decrease with time (Table 3), we also
239 present evidence that axolotls do not refuse to feed when fed an invariant diet, at least over
240 a 15 week period, arguing against the type of issues noted in some other species (Funk,
241 2006).

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

251 We urge caution when using our results because we only investigated the effects of
252 mixed diets on behavior and morphology. It is possible that dietary factors influence
253 physiological function such as immune response (Kelly & Tawes, 2013), and mixed diets
254 could have benefits here that we were unable to measure in our study. Specifically, Kelly &
255 Tawes (2013) found that female crickets fed a lower quality diet actually had better immune
256 function, presumably due to preferential investment of resources, although male crickets
257 showed no such effect. Therefore under this scenario the lower quality *Daphnia* diet may

261 higher quality diets conferred higher resistance to the pathogenic chytrid fungus.
262 Consequently, the influence of a mixed diet on aspects of health and welfare other than
263 those considered here remain unknown in axolotls, although our study still provides
264 evidence from a morphological/developmental and behavioral perspective.

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

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

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

296

297 **Acknowledgements**

298 The authors thank B. McGrath for sourcing and obtaining the study animals for the
299 experiments. We dedicate this study to 'Tiny', a very charismatic axolotl.

300

301 **Ethical Statement**

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

308

309 **Conflict of Interest Statement**

313 **Author Contributions**

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

317

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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

410

411

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415 growth over the course of the experiment. Effects of diet treatments were estimated as
 416 contrasts to the mixed diet. N=359.

417

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

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419

423 any behavior over the course of the experiment. N=718.

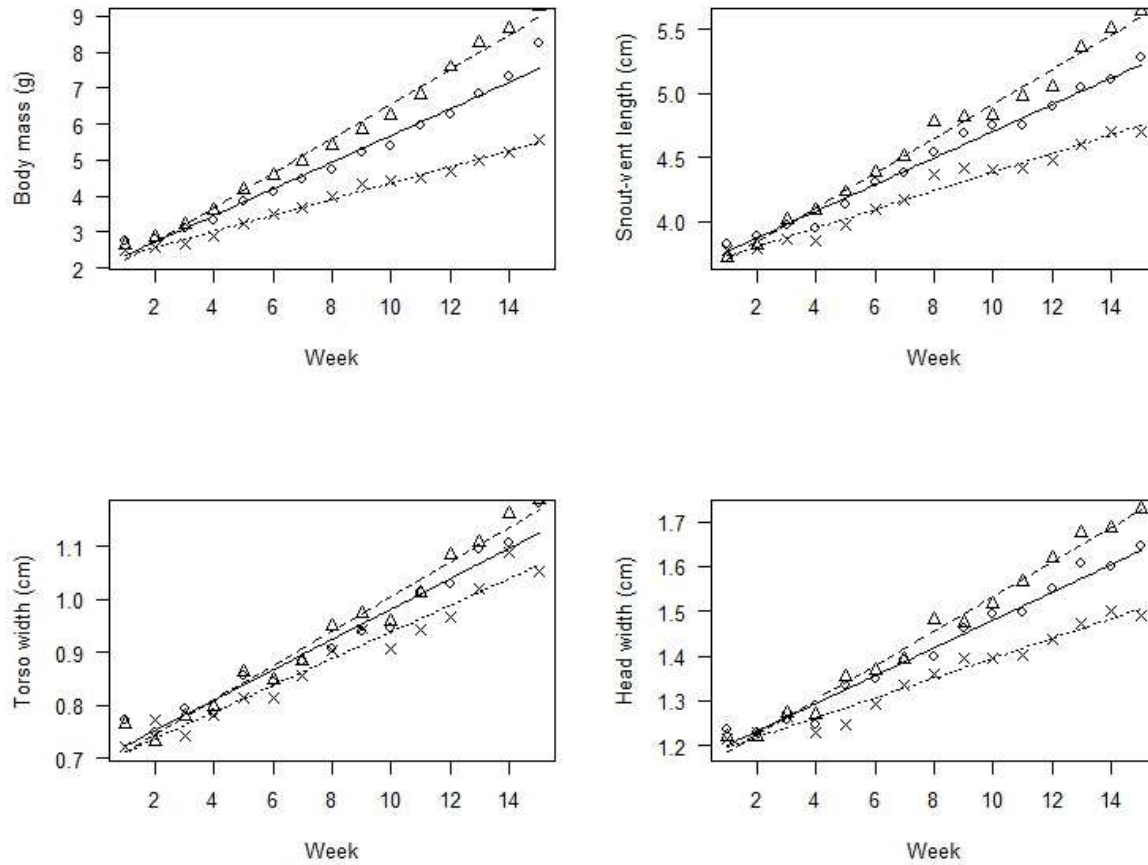
424

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

425

429 a bloodworm diet, solid lines and circles represent a mixed diet, solid lines and crosses
430 represent a *Daphnia* diet. Axolotls fed an invariant bloodworm diet grew fastest, followed by
431 those fed a mixed diet, and *Daphnia*-fed individuals grew slowest.

432

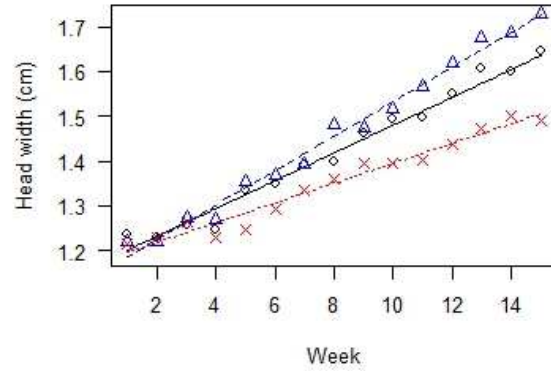
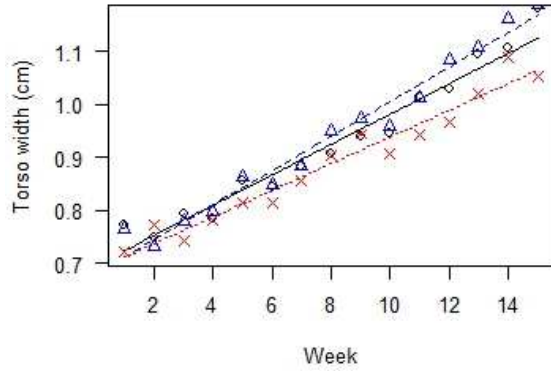
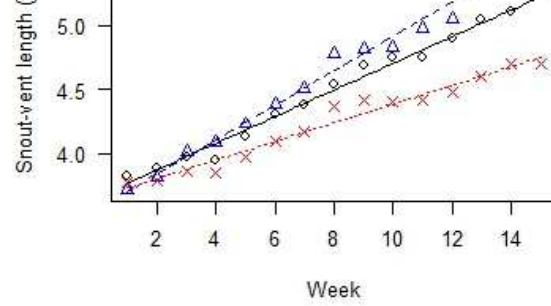
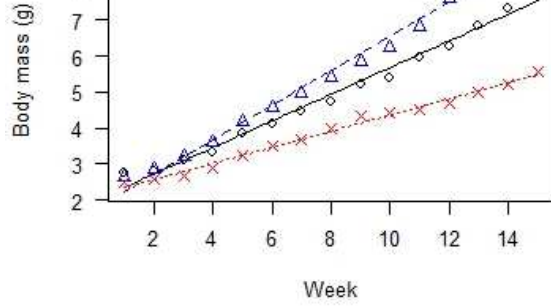


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435 GREYSCALE VERSION FOR PRINT PUBLICATION (FIGURE 1)

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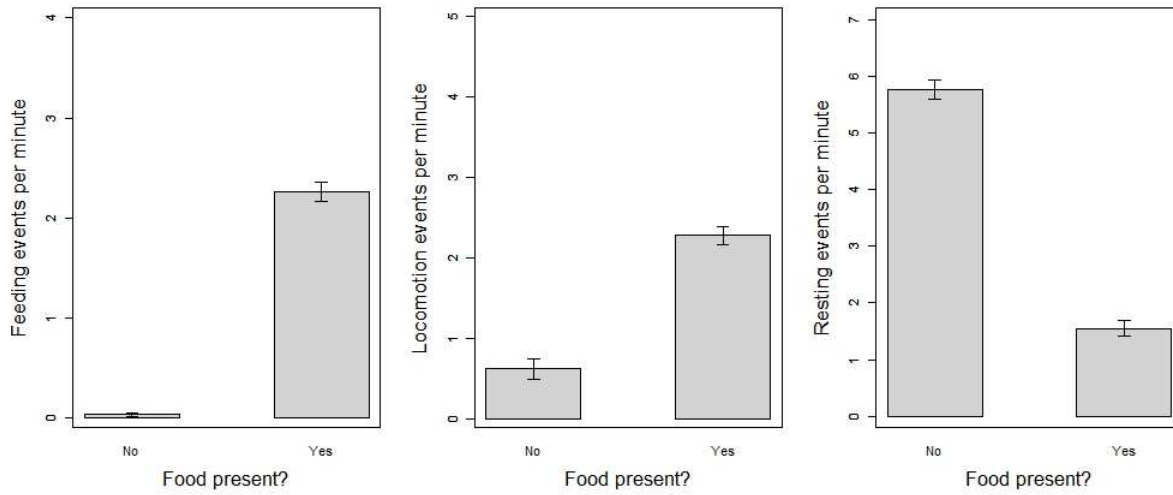
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439 COLOR VERSION FOR ONLINE PUBLICATION (FIGURE 1)

ACCEPTED

443 per minute are based on scan samples taken at 10 second intervals over one minute per
444 individual (i.e. 7 samples per minute).

445



446

increase activity.

- Despite common perceptions, mixed diets do not necessarily provide improved welfare compared to invariant diets.

ACCEPTED MANUSCRIPT