

Offspring dependence on parental care and the role of parental transfer of oral fluids in burying beetles

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40

41 **Abstract**

42

43 **Background**

44 Immature stages of many animals can forage and feed on their own, whereas others depend on their
45 parents' assistance to obtain or process food. But how does such dependency evolve, and which
46 offspring and parental traits are involved? Burying beetles (*Nicrophorus*) provide extensive biparental
47 care, including food provisioning to their offspring. Interestingly, there is substantial variation in the
48 reliance of offspring on post-hatching care among species. Here, we examine the proximate
49 mechanisms underlying offspring dependence, focusing on the larvae of *N. orbicollis*, which are not
50 able to survive in the absence of parents. We specifically asked whether the high offspring
51 dependence is caused by (1) a low starvation tolerance, (2) a low ability to self-feed or (3) the need
52 to obtain parental oral fluids. Finally, we determined how much care (i.e. duration of care) they
53 require to be able to survive.

54

55 **Results**

56 We demonstrate that *N. orbicollis* larvae are not characterized by a lower starvation tolerance than
57 larvae of the more independent species. Hatchlings of *N. orbicollis* are generally able to self-feed, but
58 the efficiency depends on the kind of food presented and differs from the more independent species.
59 Further, we show that even when providing highly dependent *N. orbicollis* larvae with easy ingestible
60 liquefied mice carrion, only few of them survived to pupation. However, adding parental oral fluids
61 significantly increased their survival rate. Finally, we demonstrate that survival and growth of
62 dependent *N. orbicollis* larvae is increased greatly by only a few hours of parental care.

63

64 **Conclusions**

65 Considering the fact that larvae of other burying beetle species are able to survive in the absence of
66 care, the high dependence of *N. orbicollis* larvae is puzzling. Even though they have not lost the

67 ability to self-feed, an easily digestible, liquefied carrion meal is not sufficient to ensure their survival.
68 However, our results indicate that the transfer of parental oral fluids is an essential component of
69 care. In the majority of mammals, offspring rely on the exchange of fluids (i.e. milk) to survive, and
70 our findings suggest that even in subsocial insects, such as burying beetles, parental fluids can
71 significantly affect offspring survival.

72

73 **Keywords**

74 *Nicrophorus*, burying beetles, parental care, trophallaxis, starvation tolerance, oral secretions,
75 offspring dependence

76 **Background**

77

78 Most animals eat to acquire nutrients that are essential to fulfil the energetic needs for their growth
79 or reproduction [1, 2]. Generally, only a part of the food an individual consumes is absorbed,
80 metabolised and converted into usable energy or nutrients, i.e. “digestive efficiency” [3–6]. Digestive
81 efficiency reflects how effectively an individual can exploit food resources, and this might vary
82 between species due to different capabilities to process and absorb food when the resource is very
83 challenging, or due to different physiological requirements, irrespective of the type of food [3]. To
84 overcome these challenges, parents in various animal taxa have evolved traits to provision offspring
85 with food or to assist them with digestion. In mammals, for example, parental care is obligate.
86 Females provide milk [7, 8], which is not only rich in lipids and proteins, but also contains bioactive
87 components such as growth factors, hormones, or immunological factors that contribute to the
88 development and protection of the young [9]. In pigeons, parents produce and feed their chicks ‘crop
89 milk’ that contains nutrients, minerals and growth factors [10], as well as immune-active compounds
90 such as immunoglobulins [11] and carotenoids [12]. Thus, food provisioning not only entails the
91 breakdown and pre-digestion of food, but also the transfer of important ancillary compounds.

92 Unlike mammals or birds, parental food provisioning occurs in only about one percent of
93 insect species [13]. Researchers have repeatedly suggested that when food is ephemeral (e.g.,
94 carrion, dung) or difficult to process (e.g., wood), insects are more likely to evolve some form of
95 parental care, such as facilitating feeding of offspring, or protecting both the resource and the
96 offspring from competitors, predators, or parasites [14, 15]. Wood roaches of the genus *Salganea*,
97 for example, have evolved morphological adaptations of the mouthparts to facilitate the uptake of
98 stomodeal substances via trophallaxis by the mother [16–18]. However, it is currently unclear
99 whether these substances contain wood fragments, nutrients, and/or enzymes or other chemicals
100 involved in the degradation of cellulose [19]. Generally, oral fluids exchanged by trophallaxis may
101 include proteins that are regulators of growth, development, and behavioural maturation [20, 21].

102 Alternatively, parents might pass symbionts to offspring that are essential for their survival and
103 growth. Altricial neonates of the wood-feeding cockroach *Cryptocercus punctulatus*, for example,
104 cannot directly process wood, but instead rely on the hindgut fluids of their parents to acquire
105 symbionts that are necessary for digestion (i.e. proctodeal trophallaxis) [22].

106 In contrast to wood or foliage, carrion is a highly nutritious and ephemeral resource that is
107 easily digested [14]. Dipteran females are usually the first insects to arrive at a carcass and to deposit
108 their eggs or first-instar larvae directly on top of the carrion, often in natural body openings or at
109 wound sites [23]. The maggots then, without any parental assistance, immediately start feeding on
110 the carrion at the site where they emerge. Most beetles in the family Silphidae also have larvae that
111 depend on carrion as their food, and in all genera except *Nicrophorus*, the larvae feed independently.
112 Only in *Nicrophorus* do adult beetles bury carcasses in an apparent attempt to monopolize and
113 defend them for their young. In addition, parents provide food to their offspring and within this
114 genus, there is significant variation in the dependence of offspring on post-hatching parental care,
115 most likely on parental feeding [24, 25]: The larvae of some species can easily feed and survive on a
116 carcass without parental help, whereas others cannot. However, the evolutionary causes driving
117 these differences in the dependence on parental care among species that utilize the same food
118 resource remain obscure. One mathematical model for the evolution of parental care predicts that in
119 species that provide care to their offspring, food provisioning is expected to evolve if it is more
120 efficient than offspring self-feeding, or more efficient than parental efforts to guard against
121 predators [26]. Also, the evolution of food provisioning promotes a mutual reinforcement between
122 parental feeding and sibling competition, resulting in a unidirectional trend from no to full parental
123 food provisioning [26]. Once parental feeding has evolved, coadaptation between parental and
124 offspring traits may lead to the delayed ontogenetic development of traits that are necessary for
125 offspring self-feeding [25].

126 However, to better understand which factors drive the evolutionary loss of independence, it
127 is crucial to determine the proximate cause of offspring dependence. On what parental service do

128 offspring actually rely, and which offspring traits differ between dependent and independent
129 species? Are there insect hatchlings that have lost their ability to self-feed similar to neonates of
130 altricial mammals and birds?

131 Here, we examine the proximate mechanisms underlying offspring dependence using burying
132 beetles as a model system. Burying beetles are well-known for their habit of interring small
133 vertebrate carcasses and providing extensive biparental care to their offspring before and after
134 hatching [27–30]. In *N. vespilloides*, larvae are capable of self-feeding, but nevertheless beg for
135 regurgitated pre-digested carrion from their parents [31, 32]. Parental regurgitations are
136 hypothesized to ensure sufficient food supply for larvae, when their mandibles are still soft and not
137 fully sclerotized following larval moults [27]. In our previous study, we found that offspring of the
138 three species *N. orbicollis*, *N. pustulatus*, and *N. vespilloides* show marked differences in their
139 dependence on parental provisioning, or at least on post-hatching care [25]. *N. orbicollis*, which is
140 one of the most basal species within the genus *Nicrophorus* [33], appears to be a beetle with
141 obligatory parental care, as offspring do not survive in the absence of parents, whereas parental care
142 is facultative in *N. pustulatus* and *N. vespilloides* [24, 25]. Likewise, parental care appears to be
143 facultative in many other *Nicrophorus* species, including *N. mexicanus* [34], *N. defodiens*, *N.*
144 *tomentosus* [24] and *N. quadripunctatus* [35]. This raises the question, therefore, as to why *N.*
145 *orbicollis* is so exceptional among other *Nicrophorus* species with regard to offspring dependency,
146 and more particularly, what causes the striking helplessness of offspring in the absence of parents. As
147 our study aimed to investigate the proximate mechanisms of offspring dependency, we focused on
148 the most dependent species studied to date, *N. orbicollis*, and drew comparisons to more
149 independent species when required.

150 We first tested the hypothesis that larvae of the different species are equally efficient at self-
151 feeding, but that *N. orbicollis* parents may invest fewer resources into eggs or their larvae may be
152 fast metabolisers. Both scenarios would result in larvae with a higher food demand in a shorter time
153 period. To test this hypothesis, we did not measure nutrient content in eggs nor the metabolic rate

154 of larvae, but rather used starvation tolerance of larvae as a proxy for a high resource need per time
155 unit. In addition, we noted larval mass at hatching as an indicator of egg investment. In the next step,
156 we investigated whether *N. orbicollis* larvae are capable of self-feeding from the time of hatching, or
157 whether the expression of morphological adaptations needed for self-feeding is delayed compared to
158 more independent species. Additionally, we tested whether the self-feeding capacity of larval *N.*
159 *orbicollis* is inferior to the self-feeding capacity of the more independent species. In burying beetles,
160 parental food provisioning entails not only the regurgitation of partially digested carrion, but also the
161 transfer of oral fluids, which might also contain important microbial symbionts, enzymes, or growth
162 hormones [see e.g. 20]. Thus, upon discovering that larval *N. orbicollis* are particularly effective in
163 feeding on pieces of baby mice, we tried to rear them on an easily digestible diet of liquefied carrion
164 either supplemented with parental oral secretions or not. With this experiment, we also tested the
165 hypothesis that the characteristics of larval mandibles play an important role in determining
166 offspring dependence. In *N. vespilloides*, a previous study has shown that larvae that receive at least
167 12 hours of parental care survive well, and average larval mass does not significantly increase with
168 longer care [28]. In a final experiment, we therefore attempted to determine the minimum duration
169 of post-hatching care required for larval survival to adulthood in *N. orbicollis*.
170

171 **Methods**

172
173 **Origin and maintenance of experimental beetles**
174 *N. vespilloides* used in the study were descendants of beetles collected from carrion-baited pitfall
175 traps in a forest near Ulm, Germany (48°25'03"N, 9°57'45"E). Cultures of *N. pustulatus* and *N.*
176 *orbicollis* were established at Ulm University from outbred colonies maintained at the Institute of
177 Zoology, University of Freiburg, Germany. We maintained outbred colonies of both species by
178 introducing beetles captured in baited pitfall traps established in a forested area near Lexington,
179 Illinois, U.S.A. (40°39'57"N, 88°53'49"W). All beetles were held in temperature-controlled incubators

180 at 20 °C on a 16:8 h light:dark cycle. Before the experiments, groups of up to five adult beetles of the
181 same sex and family of each species were kept in small plastic containers (10 x 10 cm and 6 cm high)
182 filled with moist peat. Beetles were fed freshly decapitated mealworms ad libitum twice a week. At
183 the time of experiments, beetles were virgin and between 20 and 30 days of age.

184

185 **Experimental design**

186 **Experiment 1: Starvation tolerance of larvae**

187 Larval *N. orbicollis* do not survive in the absence of post-hatching care [24, 25]. In this experiment,
188 we measured starvation tolerance of *N. orbicollis* offspring in comparison with the more independent
189 species, *N. pustulatus* and *N. vespilloides*. For this, we randomly selected non-sibling pairs of male
190 and female beetles, placed them in small plastic containers filled with peat (10 x 10 cm and 6 cm
191 high), and induced reproduction by providing them with a 20 g (± 3 g) thawed mouse carcass
192 (Frostfutter.de – B.A.F Group GmbH, Germany). In the case of the nocturnal species, *N. orbicollis* and
193 *N. pustulatus*, mice were provided during the dark portion of the photoperiod, whereas for
194 crepuscular *N. vespilloides*, mice were provided during the light portion. To prevent hatching larvae
195 from access to food, we transferred parents and the carcass to new boxes filled with peat after the
196 egg-laying period [see 25], and left the eggs to hatch in the old container. From the expected time of
197 larval hatching, we checked for larvae every hour. Then, for each species, we set up a minimum
198 number of 30 larvae from at least six different families (number of larvae, families: *N. orbicollis*: 36, 6;
199 *N. pustulatus*: 33, 11; *N. vespilloides*: 39, 9) to avoid any family effect on larval survival. One larva
200 each was added on top of a moistened paper tissue in a shallow plastic tray (3 x 3 cm x 0.5 cm high)
201 without access to food, and kept in a temperature-controlled room at 20 °C. We then checked for the
202 survival of each larva every hour up to a maximum of 42 hours, and moistened the paper tissue, if
203 necessary, to ensure an adequate supply of water to the larvae. Finally, we recorded the number of
204 hours that larvae survived.

205

206 **Experiment 2: Self-feeding ability on different food resources**

207 Here, we set up non-sibling pairs of beetles as in experiment 1 for the three species, *N. orbicollis*, *N.*
208 *pustulatus*, and *N. vespilloides*. As before, parents and their carcass were transferred to new boxes
209 after the egg laying period, and the old boxes were checked at least every 8 hours for the hatching of
210 larvae. To measure interspecific variation in the ability of larvae to self-feed when parents are
211 absent, we established three treatments per species ($n = 15$ for each species and treatment) in which
212 we offered individual larvae one of three different food resources ranging from very challenging food
213 to very easily accessible and digestible food: (1) carrion prepared by parents in the pre-hatching
214 period without a hole created by parents; (2) carrion prepared by parents in the pre-hatching period
215 with a hole in the carcass created by parents; (3) small pieces of baby mice. Generally, burying beetle
216 parents create an opening in the carcass shortly before or after larval hatching, allowing larvae direct
217 access to the food [28]. To ensure that we obtained approximately equal numbers of prepared
218 carcasses with ($n = 15$) and without a hole ($n = 15$), we set up additional pairs for reproduction in
219 each species. Thus, for treatments 1 and 2, we provided 50 pairs in each species with a 20 g (± 3 g)
220 thawed mouse carcass and allowed them to provide pre-hatching care according to their species-
221 specific duration (*N. orbicollis*: 120 h; *N. pustulatus*: 80 h; *N. vespilloides*: 70 h). We then inspected
222 prepared carcasses for an opening in the integument and assigned them to the treatment “prepared
223 carrion without hole” in those instances where there was no hole. Carcasses that had already been
224 processed and opened by the parents were additionally cut open using scissors and assigned to the
225 treatment “prepared carrion with hole”. All food resources were offered in small plastic containers
226 without peat (10 x 10 cm and 6 cm high), but lined with moist paper tissue. As soon as the larvae
227 hatched, their initial mass (0 h) was determined to 0.01 mg using a precision scale (Kern ABT 220-
228 5DM, Kern und Sohn GmbH, Balingen, Germany) before allowing them access to a food resource.
229 Immediately thereafter, one larva each was randomly added on top of one of the three food
230 resources. Larvae were then weighed again 2 hours later to detect any changes in larval mass during
231 this time interval.

232

233 **Experiment 3: Effect of oral secretions on larval *N. orbicollis***

234 Here, we determined whether larvae of the most dependent species, *N. orbicollis*, could be reared in
235 the absence of their parents when provided with a liquidized paste of baby mice mixed with or
236 without oral fluids of their parents. For this, we established 40 pairs of male and female beetles, 20
237 of which were set up 2 days in advance and used for the extraction of oral secretions. The other 20
238 pairs served to provide larvae for the actual experiment. As before, parents and the carcass were
239 transferred to new boxes after the egg-laying period, leaving the eggs in the old boxes to hatch. We
240 established two treatment groups in which we provided larvae with (1) a paste of baby mice that
241 included oral secretions of care-giving male or female parents that had been given access to larvae
242 and a carcass for 24 - 48 hours (n = 35), or (2) a paste of baby mice without oral secretions of parents
243 (n = 35). To prepare the paste of baby mice, we placed 30 dead and frozen baby mice (1 - 3 g;
244 Frostfutter.de—B.A.F Group GmbH, Germany) into a blender together with 30 mL of water, and
245 mixed them until the paste was homogenous. To obtain the regurgitated oral fluids from a parent,
246 we gently squeezed the thoracic-abdominal region of a beetle with a pair of forceps and collected
247 the secretions with a pipette. For the experiments, we placed 5 larvae from one family (n = 7 for each
248 treatment) that had hatched at the same time together in a petri dish containing a moist paper
249 tissue. We then checked for the survival of larvae three times a day, and exchanged both the moist
250 paper tissue and the food when larvae were still alive. We recorded the number of hours each larva
251 survived.

252 In the group including oral secretions of parents (1), larvae were provided with 5 µL of oral
253 secretions directly added on top of the moist paper tissue for the first 24 hours. In addition, we
254 added two 0.2 mL Eppendorf tubes containing 5 mg of baby mouse paste mixed with 5 µL of oral
255 secretions of a parental beetle. Oral secretions were always obtained freshly from the parental
256 beetles. In the group without oral secretions (2), larvae in the first 24 hours were only provided with
257 two 0.2 mL Eppendorf tubes that contained approximately 5 mg of baby mouse paste. The Eppendorf

258 tubes in both treatments were sliced open at both ends to facilitate ready access of larvae to the
259 food. After 24 hours, larvae of both treatments received one 0.5 mL Eppendorf tube containing baby
260 mouse paste without oral secretions. After 48 hours, larvae received one opened baby mouse
261 carcass and one 0.5 mL Eppendorf tube containing baby mouse paste without oral secretions. As
262 parental regurgitations in *N. orbicollis* substantially decrease after 48 hours, and larval survival and
263 mass are subsequently not reduced in the absence of care [36, 37, see also experiment 4], we opted
264 to provide larvae solely with mouse carcasses thereafter. After 120 hours, surviving larvae were
265 placed into boxes with soil and provided with two opened baby mouse carcasses in succession, the
266 first of which was left for 8 hours, after which it was exchanged with the second carcass for an
267 additional 8 hours. This was done to ensure a sufficient food supply for larvae just prior to pupation.
268 After the second carcass was removed, the larvae were left to pupate.

269

270 **Experiment 4: Duration of post-hatching care needed to ensure development of larval *N. orbicollis***
271 The aim of this experiment was to determine the minimum duration of post-hatching care needed to
272 ensure survival of larvae. To test this, we set up 200 non-sibling pairs of *N. orbicollis* beetles as in the
273 previous experiments. After the egg laying period [see 25], parents and their carcass were
274 transferred to new boxes, and the old boxes were checked every 8 hours for the hatching of larvae.
275 To control for variation between families and individual differences in behaviour [38], we provided
276 each pair of beetles with a brood of 15 newly hatched larvae of mixed parentage [25, 39, see also
277 40]. Burying beetles exhibit temporally-based kin discrimination in which they kill any larvae arriving
278 on the carcass before their own eggs would have hatched [41]. Hence, we only provided pairs with
279 larvae after their own larvae had begun hatching. The larvae were placed directly onto the carcass, in
280 which we had cut a hole through the skin earlier to facilitate larval access to the carrion in each of
281 the treatments. We then allowed parents to provide post-hatching care for 1 h, 3 h, 6 h, 12 h, 24 h,
282 or 48 h (n = 15 or 16 per treatment). In addition, we established a “pre-hatching care” treatment (n =
283 15), in which parents were only allowed to prepare the carcass, but were prevented from providing

284 post-hatching care (“0 h”). Finally, we also established a “full-care” treatment, in which parents were
285 allowed to prepare the carcass and to provide post-hatching care until larvae dispersed (8 ± 2 days).
286 As soon as the surviving larvae of each brood left the carcass for pupation, they were counted and
287 weighed.

288

289 **Statistical analysis**

290 All data were analysed and plotted using R version 3.1.2 (R Core Team 2014) or SPSS version 21.0
291 (Chicago, IL, USA). For experiments 1 and 3, we used the Kaplan-Meier method in SPSS to estimate
292 survival of larvae as a function of time. To test for differences in larval survival between the three
293 species in experiment 1 and the two treatments in experiment 3, we used a log-rank test in SPSS. For
294 experiment 2, we used the relative change in larval mass between 0 h and 2 h as a proxy to assess
295 the ability to self-feed in each species. As larval mass at hatching differed among species (GLM with
296 Gaussian errors: $F_{2,177} = 517.69, P < 0.001$), we first divided the absolute change in larval mass by the
297 mass of each larva at hatching. We then applied generalised linear models (GLMs) with Gaussian
298 distribution with species, treatment and species*treatment as fixed factors and the relative change
299 in larval mass as the dependent variable. To identify species-specific treatment effects, we continued
300 with GLMs followed by pairwise comparisons with Bonferroni correction for multiple testing within
301 each of the three species in which treatment was included as a fixed factor and the relative change in
302 larval mass as the dependent variable. For experiment 4, we included duration of post-hatching care
303 (0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, full care) as a fixed factor, and the absolute number of larvae that
304 survived and mean larval mass per brood as dependent variables. We then applied GLMs with
305 Poisson distribution followed by pairwise comparisons with Bonferroni correction for multiple
306 testing. In addition, we compared the mean larval mass per brood at dispersal by using a GLM with
307 Gaussian distribution.

308

309 **Results**

310

311 **Experiment 1: Starvation tolerance of larvae**

312 Survival of larvae without access to food varied significantly among the three species (log-rank test,
313 for all: $P < 0.001$, see Fig. 1). On average, highly dependent *N. orbicollis* larvae survived longer (mean:
314 $17.44 \pm \text{SE } 0.75$ h) than larval *N. vespilloides* (mean: $10.21 \pm \text{SE } 0.43$ h) which show an intermediate
315 dependence on parental care (log-rank test, $\chi^2 = 54.28$, $P < 0.001$). However, *N. orbicollis* larvae
316 survived significantly shorter than the highly independent *N. pustulatus* larvae (mean: $30.82 \pm \text{SE}$
317 1.04 h) (log-rank test, $\chi^2 = 62.53$, $P < 0.001$). *N. pustulatus* larvae also survived longer than larval *N.*
318 *vespilloides* (log-rank test, $\chi^2 = 77.03$, $P < 0.001$).

319

320 **Experiment 2: Self-feeding ability on different food resources**

321 When we provided individual larvae with one of three different food resources, we found significant
322 effects of treatment and species as well as a significant interaction on the relative change in larval
323 mass (Table 1, see Fig. 2). Across all treatments, highly dependent *N. orbicollis* larvae gained less
324 larval mass (mean: $0.04 \pm \text{SE } 0.02$ mg) than independent *N. pustulatus* (mean $0.16 \pm \text{SE } 0.03$ mg)
325 (Pairwise test: $P < 0.001$). There was no difference in the change in larval mass between *N. orbicollis*
326 and *N. vespilloides* (mean $0.06 \pm \text{SE } 0.01$ mg) (Pairwise test: $P = 1.00$). The gain in larval mass was
327 higher in *N. pustulatus* than in *N. vespilloides* (Pairwise test: $P = 0.003$). The type of food had an
328 effect on change in larval mass, but this effect differed among the species (Table 1, see Fig. 2). *N.*
329 *orbicollis* and *N. pustulatus* exhibited increased mass when provided with baby mice, whereas larval
330 *N. vespilloides* did not. Also, cutting a hole into the carrion had a clear positive effect on weight
331 increase in larval *N. pustulatus*, but not in the other two species.

332

333

factor	relative change in larval mass		
	df	F	P

species	2	13.44	< 0.001
treatment	2	17.61	< 0.001
species*treatment	4	6.00	< 0.001

334

335 **Table 1:** Results of the GLM of the effect of species (*N. orbicollis*, *N. pustulatus*, *N. vespilloides*),
 336 treatment (prepared carrion without hole, prepared carrion with hole, baby mice, overall sample size
 337 = 135) and the interaction of species and treatment on relative change in larval mass. Significant p-
 338 values are typed in bold.

339

340 To better understand how the type of food affected the relative change in larval mass, we
 341 analysed each species separately. We found that the change in larval mass depended on the food
 342 provided in *N. orbicollis* and *N. pustulatus* (GLM with Gaussian errors: $F_{2,42} = 20.52$, $P < 0.001$ for *N.*
 343 *orbicollis*; $F_{2,42} = 10.10$, $P < 0.001$ for *N. pustulatus*), but not in *N. vespilloides* (GLM with Gaussian
 344 errors: $F_{2,42} = 1.36$, $P = 0.27$). In *N. orbicollis*, larvae only gained weight when provided with baby mice
 345 (Pairwise test: $P < 0.001$ for baby mice vs. prepared carrion with hole, baby mice vs. prepared carrion
 346 without hole, Fig. 3A). In contrast to highly dependent *N. orbicollis*, larvae of the more independent
 347 species were able to gain weight when provided with a carcass that was prepared by the parents. In
 348 *N. pustulatus*, larvae showed a greater increase in mass when provided with a prepared carcass with
 349 a hole (Pairwise test: $P = 0.01$) or baby mice than when provided with a prepared carcass without a
 350 hole (Pairwise test: $P < 0.001$, Fig. 3B). Larval *N. vespilloides* gained weight equally on the different
 351 types of food (see Fig. 3C).

352 Further, we should note that larval mass at hatching differed significantly among the three
 353 species (GLM with Gaussian errors: $F_{2,177} = 517.69$, $P < 0.001$). On average, larval *N. vespilloides* were
 354 heavier than larval *N. orbicollis* and larval *N. pustulatus* at hatching (Pairwise test: for both, $P <$
 355 0.001). Larval *N. orbicollis* were, in turn, heavier than larval *N. pustulatus* (Pairwise test: $P < 0.001$).

356

357 **Experiment 3: Effect of oral secretions on larval *N. orbicollis***

358 *N. orbicollis* larvae receiving baby mouse paste with oral secretions from parental beetles survived
359 significantly longer than larvae that received plain baby mouse paste (log-rank test, $\chi^2 = 4.30$, $P =$
360 0.038, see Fig. 4). On average, larvae that received baby mouse paste without oral secretions
361 survived 43.84 (± 3.39) hours, whereas larvae receiving baby mouse paste mixed with oral secretions
362 survived 58.27 (± 6.37) hours on average. Five out of 35 larvae fed baby mouse paste with oral
363 secretions pupated, but only one of 35 larvae fed plain baby mouse paste did.

364

365 **Experiment 4: Duration of post-hatching care needed to ensure independence in larval *N. orbicollis***

366 Out of a total of 1815 *N. orbicollis* larvae, 951 survived. Larval survival was significantly affected by
367 the duration of post-hatching care that parents provided (GLM with Poisson errors: $F_{7,113} = 44.69$, $P <$
368 0.001, see Fig. 5A). We found that 3 h of care significantly increased the number of larvae that
369 survived to dispersal compared to broods that received 0 h (Pairwise test: $P < 0.001$) or 1 h of care
370 (Pairwise test: $P = 0.006$). When considering the number of broods in which some larvae survived, 1
371 hour of care is not yet sufficient to significantly increase survival rate (broods with/without surviving
372 larvae: 0 h: 0/15; 1 h: 4/11; Fisher's exact test: $P = 0.100$). However, it is clear that 3 hours of care is
373 sufficient to increase survival rate substantially; 87.5 % of the broods had surviving larvae after only 3
374 hours of parental attendance compared to none with 0 h of care (broods with/without surviving
375 larvae: 0 h: 0/15; 3 h: 14/2; Fisher's exact test: $P < 0.001$). In fact, 3 hours of parental care did not
376 differ from full care in terms of larval survival (Pairwise test: $P = 1.00$).

377 The duration of post-hatching care had a significant effect on larval mass (GLM with Gaussian
378 errors: $F_{6,86} = 6.31$, $P < 0.001$, see Fig. 5B). The longer larvae received post-hatching care, the heavier
379 they were when dispersing from the carcass. Within all surviving broods, larvae were significantly
380 heavier when receiving 48 h of post-hatching care or full care than when receiving 3 h (Pairwise test:
381 48 h, $P = 0.003$; full care, $P = 0.002$) or 6 h of care (Pairwise test: 48 h, $P = 0.009$; full care, $P = 0.005$).

382 As no larvae survived in the pre-hatching care treatment (0 h), we could not include this treatment in
383 this analysis.

384

385 **Discussion**

386

387 The results of our study reveal new insights into the proximate causes of the extreme dependence of
388 *N. orbicollis* offspring on parental care, and the variation in offspring dependence among species. We
389 found that starvation tolerance of larvae varied among species, but did not appear to be related to
390 dependence on parental care. Newly hatched *N. orbicollis* were generally able to self-feed, but the
391 capacity for utilizing different types of food was more limited than in the more independent species,
392 *N. pustulatus* and *N. vespilloides*. Dependent *N. orbicollis* gained less weight when self-feeding than
393 nutritionally independent *N. pustulatus*. In addition, our study revealed that even a highly processed
394 liquefied carrion meal is not sufficient to secure larval survival in *N. orbicollis*; however, oral
395 secretions of parents mixed into a purée of baby mice prolong the survival of larval *N. orbicollis*
396 without parents, but not long enough for most larvae to pupate. Finally, we revealed that three
397 hours of post-hatching care was sufficient to achieve a significant increase in the survival and final
398 mass of the larvae of the most dependent species, *N. orbicollis*. Our results highlight key
399 characteristics of offspring and parental traits that augment our understanding of offspring
400 dependence on parental care. Below, we elaborate on the wider implications of these results.

401 The results of our first experiment make it unlikely that starvation tolerance is related to high
402 levels of offspring dependence, but is instead more likely associated with variation in growth rate.
403 Here, we tested whether larvae of the three species, *N. orbicollis*, *N. pustulatus*, and *N. vespilloides*,
404 differ in their tolerance to starvation in the absence of parents. Combined with information on egg
405 investment, represented by mass at hatching (see Fig. 3), the level of starvation tolerance could
406 provide information on whether larvae are fast or slow metabolisers, or on the parental investment
407 in egg composition, and could thus be related to the marked offspring dependence on parental

408 provisioning in *N. orbicollis*. As expected, we found that offspring of the more independent species,
409 *N. pustulatus*, were most tolerant to starvation and survived the longest in the absence of food.
410 Surprisingly, however, the highly dependent larvae of *N. orbicollis* starved to death later than larvae
411 of *N. vespilloides*, which show an intermediate dependence on parental care [25]. Here, hatchlings of
412 *N. vespilloides* were the heaviest, followed by hatchlings of *N. orbicollis*, and then *N. pustulatus*, the
413 lightest of the three species [but see 42]. Given their low mass at hatching, it is even more striking
414 that most of the larval *N. pustulatus* were still alive when larvae of the other two species had all
415 starved to death, suggesting that *N. pustulatus* are slow metabolisers. Generally, starvation
416 resistance tends to increase with body size and larger energy stores, despite the greater absolute
417 energy needs of larger individuals [43]. However, larval *N. vespilloides* are not only the heaviest at
418 hatching, but also have the highest growth rate of the three species during the first 48 hours with full
419 care [25]. Faster growth rates are usually associated with a greater need for food and higher
420 metabolic rates, making fast-growing individuals, such as *N. vespilloides*, more vulnerable to
421 starvation when resources are limited [44].

422 The aim of our second experiment was to investigate whether hatchlings of *N. orbicollis* are
423 able to self-feed, or whether traits necessary for self-feeding only develop at a later larval stage
424 compared to the more independent species, which might explain strong offspring dependency on
425 parental care. Larvae of passalid beetles, for example, differ in their ability to feed themselves and to
426 construct feeding tunnels, and in their dependency on parental care [14]. Here, we found that newly
427 hatched larvae of *N. orbicollis* were generally able to self-feed and gain weight when reared on baby
428 mice, but not on prepared carcasses that parents usually use as a food resource for their offspring in
429 nature. In contrast, no clear pattern was found in the two more independent species as larvae also
430 increased in mass when provided with parent-prepared carcasses. It might not be surprising that
431 larvae gain more weight on pieces of baby mice than, for example, on prepared carrion. First, baby
432 mice are younger and probably have a higher water content, but fewer hard body parts than adult
433 mice, making them more easily accessible for larvae. Second, pieces of baby mice are certainly

434 fresher than the parent-prepared carrion. Further, the larvae of different species obviously differ in
435 their ability to access and process different types of vertebrate carrion, which could be related to
436 quantitative or qualitative differences in the oral digestive enzymes of larvae. It may be that the
437 digestive system of young *N. orbicollis* hatchlings has evolved to rely more on pre-digested food from
438 parents at the beginning, and later on, the slightly older larvae become able to consume and
439 assimilate solid food on their own.

440 Alternatively, larval ability to self-feed might depend on species-specific characteristics of the
441 mandibles. It is conceivable that mouthparts of *N. orbicollis* larvae may develop and sclerotize at a
442 slower rate than the mouthparts of the other two species in our study, resulting in less robust
443 mandibles that do not allow larvae to self-feed initially. Pukowski [27] observed that hatchlings and
444 recently moulted larvae of *N. vespillo* are unable to self-feed, and ascribed this to their unsclerotized
445 mouthparts. Only after five to six hours, were larvae observed to self-feed [27]. Thus, differences in
446 the sclerotization rate of mandibles could contribute to the variation in self-feeding and offspring
447 dependence on parental care. In species with obligatory parental care, such as *N. orbicollis*, selection
448 on mandible sclerotization rate or other traits, such as the production of digestive enzymes that
449 could facilitate nutritional independence of offspring, may be relaxed as parents assume a greater
450 share of the services related to food intake. As the expression and maintenance of these traits is
451 generally costly [45], traits related to self-feeding may only be expressed later in life when parents
452 withdraw from providing parental care and offspring need to become independent. Generally, as
453 soon as offspring traits are no longer in use because parents take over the tasks that secure offspring
454 survival by providing parental care, a reduction in the relevant offspring traits is expected. This
455 reduction, in turn, further drives the evolution of increased offspring dependency on parental care.
456 For example, first instar neonates of wood-feeding *Cryptocercus* cockroaches, which exhibit
457 elaborate biparental care, completely lack eyes and have a pale and thin cuticle [16]. The hindgut
458 symbionts that help larvae to metabolise and digest wood are not fully established until the third
459 larval instar [46]. Consequently, until that time, nymphs depend on their parents for nutrition and

460 symbiont transfer [16]. Like *Cryptocercus*, first instar larvae of wood-feeding *Salganea* have a pale
461 and transparent cuticle, and their eyes are present, but considerably reduced [16]. Larvae feed on
462 parental oral fluids and are somewhat less dependent than larval *Cryptocercus*, but more dependent
463 than *Panesthia* neonates that are well developed and show no interactions with parents [16]. In
464 these three genera, the developmental characteristics of neonates appear to parallel a gradient of
465 dependence on parental care [16].

466 Eggert et al. [28] showed that 12 hours of parental care resulted in a significant increase in
467 survival and growth of larval *N. vespilloides*, suggesting that this was due, in part, to the opening in
468 the carcass that is created by the parents, thereby facilitating easier access of the larvae to the
469 carrion. In an experimental evolution study, larvae descended from beetles reared in the absence of
470 post-hatching care became increasingly independent, a result that was attributed to the ability of
471 larvae to self-feed more efficiently or through morphological adaptation of larval mouthparts [47].
472 Although these behavioural or morphological adaptations are undoubtedly advantageous, their
473 absence in larval *N. orbicollis* alone cannot explain their nutritional dependency. In our study, even
474 an opening in the integument of a prepared carcass did not increase the efficiency of larvae to self-
475 feed. Also, although larval *N. orbicollis* were able to consume small pieces of juvenile mouse
476 carcasses, none of the larvae were able to survive more than 24 hours in the absence of parents (A.
477 Capodeanu-Nägler, pers. obs.). Even when provided with liquefied mouse carrion, most of the larvae
478 did not survive to pupation.

479 One other factor that could account for the differences in self-feeding is the behaviour of
480 larvae towards food when parents are absent. From a study on *N. vespilloides*, we know that larvae
481 cooperate to penetrate the carcass when parents are absent [48]. One precondition for cooperation
482 between siblings is that larvae need to aggregate first. Generally, larvae seem to be attracted to one
483 another and without another larva, larvae of the more independent species may have directly
484 attempted to feed. However, larvae of *N. orbicollis* that benefit most from their parents' care, might
485 be selected to focus on approaching their parents instead of converging to other larvae. Thus,

486 especially when carcass preparation indicates the presence of parents by parent-derived cues on the
487 carcass surface, larvae might wander around and search for a parent instead of attempting to feed
488 (A. Capodeanu-Nägler, pers. obs.). Nevertheless, behavioural observations are needed to confirm
489 these predictions.

490 Having shown that highly dependent *N. orbicollis* larvae are able to self-feed and increase in
491 weight when provided with small pieces of baby mice, we attempted to determine whether they
492 could be successfully reared in the absence of parents on a diet of homogenized mouse carrion
493 mixed with oral secretions from parental beetles. We found that larvae reared on this diet were
494 more likely to survive to dispersal than larvae receiving the same diet but without parental
495 secretions. Thus, oral secretions of parents are clearly beneficial to *N. orbicollis* larvae, and may
496 contain important symbionts, antimicrobial compounds, enzymes, or hormones. Eggert et al. [28]
497 examined the importance of symbiont transfer in *N. vespilloides*, but found that the positive effects
498 of parental provisioning on larval survival and growth were not mediated by the transfer of
499 symbionts. However, the transfer of symbionts in *N. orbicollis* may be more important as larvae in
500 this species are more dependent on parental provisioning. In addition, the beetles' anal and oral
501 secretions contain a wide range of compounds, some of which have antimicrobial properties [49–51],
502 and express a variety of immune-related genes [52] with several antimicrobial peptides and
503 lysozymes that are specifically upregulated in the presence of carrion [53, 54], and that could
504 enhance offspring survival. Finally, parents may transfer growth-regulatory proteins or hormones
505 that are essential for survival and development of dependent offspring. Juvenile hormone III (JH III),
506 for example, has recently been found to be transferred to larvae by trophallaxis in ants [20]. In
507 burying beetles, JH III plays a regulatory role in a multiple contexts [55–61], and parents might thus
508 transfer some JH III when they regurgitate to larvae, which may contribute to their survival and
509 growth [but see 62].

510 Alternatively, oral secretions might signal the presence of parents to offspring. Carpenter
511 ants, for instance, have been shown to exchange chemical signals by trophallaxis that help them to

512 recognize nestmates [20, 21]. Likewise, oral secretions of burying beetles might have a signalling
513 function that helps larvae to localize pre-digested food or initiates larval feeding. Nonetheless,
514 despite receiving homogenized carrion mixed with oral secretions of parents, most of the larvae of *N.*
515 *orbicollis* did not survive until dispersal. However, since we do not know the actual volume of oral
516 fluids that parents transfer to larvae, we may have provided larvae with less than the requisite
517 amount of oral secretions. In our last experiment, we showed that larval survival and mass of *N.*
518 *orbicollis* increased with the duration of post-hatching care, which is not surprising as parental care
519 usually enhances offspring fitness [7, 8]. More surprisingly, we found that survival of the highly
520 dependent *N. orbicollis* larvae was significantly enhanced after only three hours of parental care.
521 Why might such a short period of care have such a profound effect on offspring survival? For *N.*
522 *vespilloides*, larval begging as well as parental provisioning is known to peak 24 hours after hatching
523 [32, 63]. However, we observed larvae begging and parents provisioning in the first three hours after
524 hatching (A. Capodeanu-Nägler and M. Prang, pers. obs.). Thus, parents might provide begging larvae
525 with enough food during these first few hours that larvae have sufficient energy to survive until they
526 are efficient self-feeders.

527 In the light of the other results of our study, however, we find it more likely that the transfer
528 of oral secretions and maybe also anal secretions might be crucial for larval survival and growth,
529 especially in the first few hours after larval hatching. For example, if larvae are given a single dose of
530 symbionts by the parents in the first three hours after hatching, they may be able to survive
531 thereafter. Burying beetles are known to harbour a diverse gut microbiome including various
532 *Yarrowia*-like yeasts [64]. *Yarrowia* are present in both adult and larval life stages, and are possibly
533 involved in carrion digestion and preservation [65]. More recent studies have shown that burying
534 beetle parents not only transfer microorganisms to larvae via oral secretions, but tightly regulate the
535 microbiome of the carcass by applying anal and oral secretions to it, which serves not only as a
536 nutritional resource, but also facilitates the vertical transmission of symbiotic microbiota to larvae
537 [66–68]. Thus, the transfer of preservation- and digestion-related microbiota to the carcass during

538 the first hours might enhance larval survival for the more dependent offspring of *N. orbicollis* after
539 parents have been removed.

540

541 **Conclusions**

542

543 Our study offers new insights into offspring and parental traits that appear to be relevant to the
544 evolution of marked offspring dependence of certain species. We showed that tolerance to
545 starvation differs greatly between species, but this is not likely to be associated with the high degree
546 of offspring dependence in *N. orbicollis*. Nevertheless, *N. orbicollis* larvae are generally able to self-
547 feed, but they are less efficient than larvae of the two more independent species. The variation in the
548 efficiency to self-feed is probably not only due to differences in the structure or strength of larval
549 mandibles, as larval *N. orbicollis* do not even survive when provided with liquefied mouse carrion for
550 which the use of mandibles is redundant. As even short periods of parental care and easily accessible
551 food containing oral secretions of parents significantly enhance survival of highly dependent *N.*
552 *orbicollis*, we conclude that parental fluids must contain symbionts or other components that are
553 crucial for larval survival. Thus, future studies should investigate the transfer and contents of oral
554 fluids from parents to offspring more closely, which will further help to understand how coevolution
555 drives an increasingly tight integration of offspring development and parental care [26, 69–73].

556 **List of abbreviations**

557 Not applicable

558

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562

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567

568 **Availability of data and materials**

569 The datasets used and/or analysed during the current study are available from the corresponding
570 author on reasonable request.

571

572 **Authors' contributions**

573 SS conceived the study. ACN and SS designed the study. ACN and MAP performed the experiments.
574 ACN and MAP analysed the data. ACN, MAP, STT, HV, AKE, SKS, and SS discussed the results. ACN
575 wrote a draft manuscript and MAP, STT, HV, AKE, SKS, and SS contributed substantially to revision. All
576 authors read and approved the final manuscript.

577

578 **Ethics approval and consent to participate**

579 No human subjects were involved in the study. All work on this project was done in accordance with
580 state and federal regulations with respect to maintenance and handling of burying beetles. The

581 burying beetle species *N. orbicollis*, *N. pustulatus*, and *N. vespilloides* are not regulated or
582 endangered species.

583

584 **Consent for publication**

585 Not applicable.

586

587 **Competing interests**

588 The authors declare that they have no competing interests.

589

590 **Figure legends**

591

592 **Fig. 1:** Survival of larvae without access to food in *N. orbicollis* (N = 36), *N. pustulatus* (N = 33), and *N. vespilloides* (N = 39) [h]. Kaplan-Meier estimated survival curves.

594

595 **Fig. 2:** The change in larval mass from hatching to two hours after hatching in *N. orbicollis*, *N.*
596 *pustulatus*, and *N. vespilloides* on different food sources [mg]. N = 15 for each species and treatment.
597 Boxplots show median, interquartile range, minimum/maximum range. The dots are values that fall
598 outside the interquartile range ($> 1.5 \times$ interquartile range). Different letters indicate significant
599 differences between species within one treatment.

600

601 **Fig. 3:** Larval masses [mg] at hatching (0 h) and after two hours on a specific food source in (A) *N.*
602 *orbicollis*, (B) *N. pustulatus* and (C) *N. vespilloides*. N = 15 for each species and treatment. Each line
603 represents one individual larva. The red line represent the mean of all larvae in one treatment.

604

605 **Fig. 4:** Survival of *N. orbicollis* larvae that received a paste of baby mice with or without oral
606 secretions of parents [h]. N = 7 x 5 larvae for both treatments. Kaplan-Meier estimated survival
607 curves.

608

609 **Fig. 5:** (A) Percent of larvae surviving to dispersal and (B) Mean larval mass per brood at dispersal
610 [mg] of *N. orbicollis* larvae that received different durations of post-hatching care before parents
611 were removed. N = 15 or 16 per treatment. Boxplots show median, interquartile range,
612 minimum/maximum range. The dots are values that fall outside the interquartile range ($> 1.5 \times$
613 interquartile range). Different letters indicate significant differences between treatments.

614

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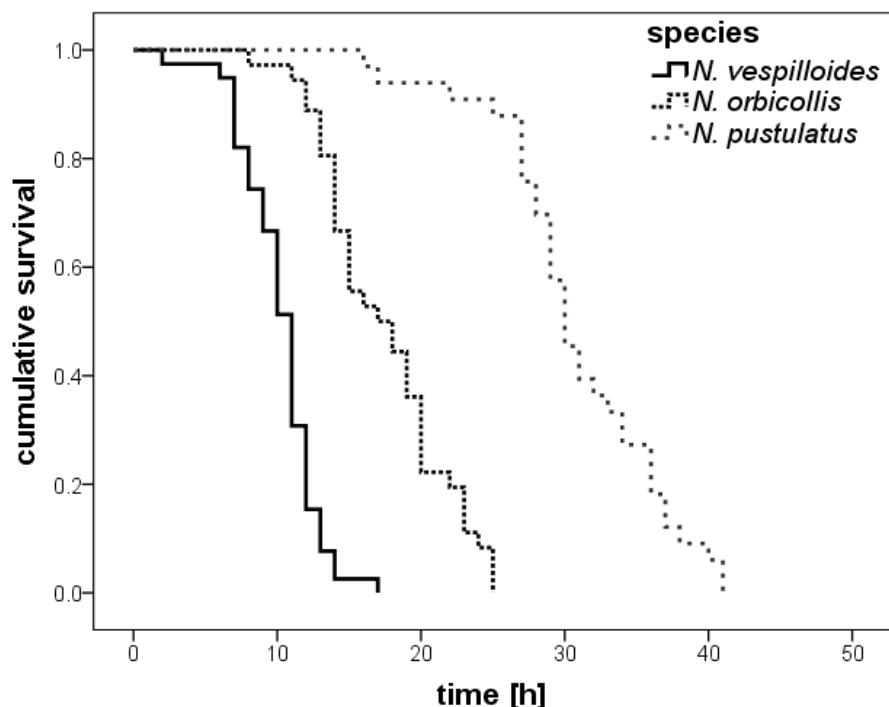
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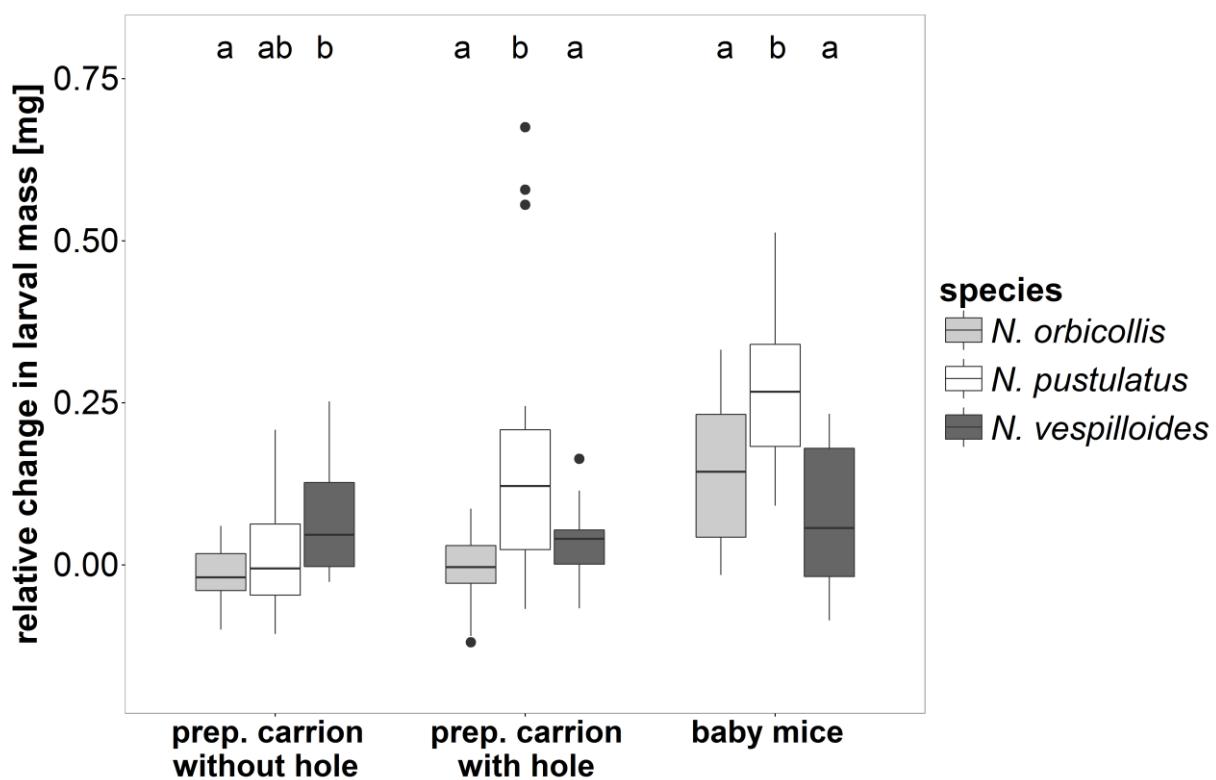
774 **Fig. 1**



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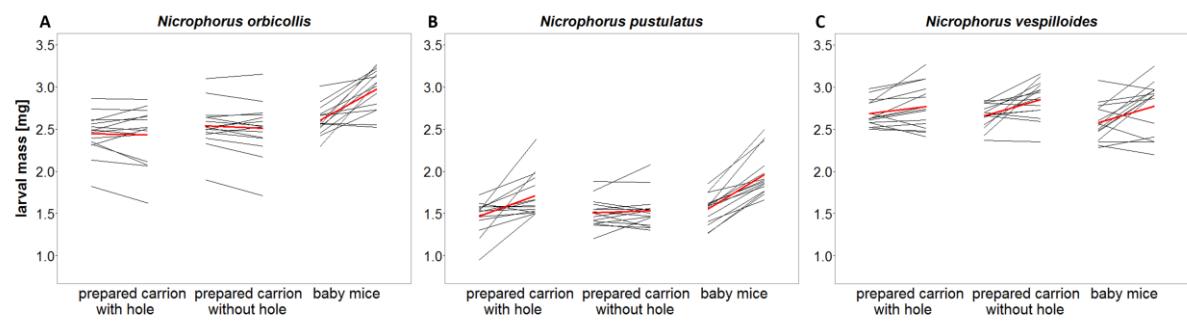
777 **Fig. 2**



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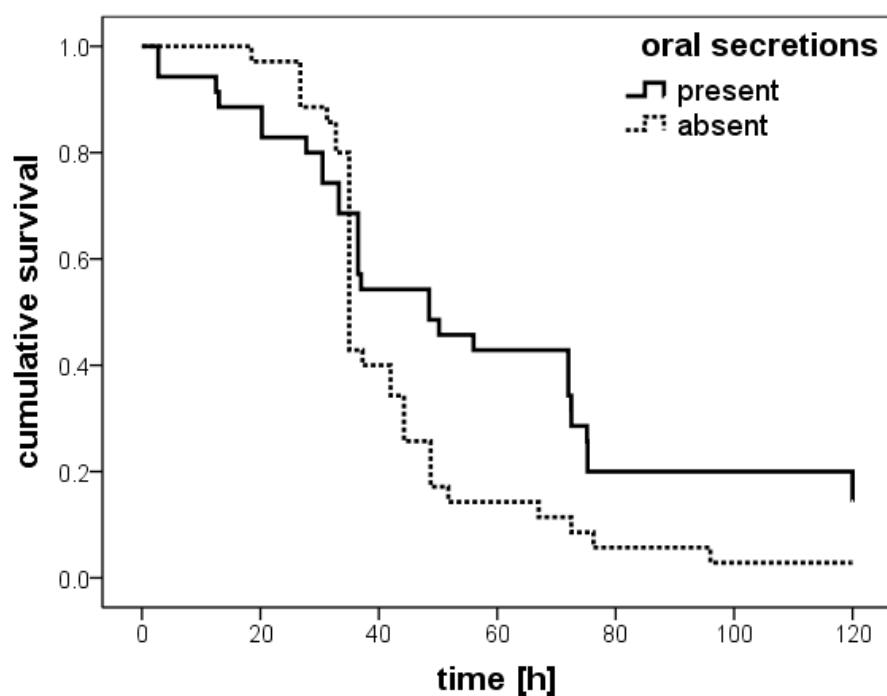
780 **Fig. 3**



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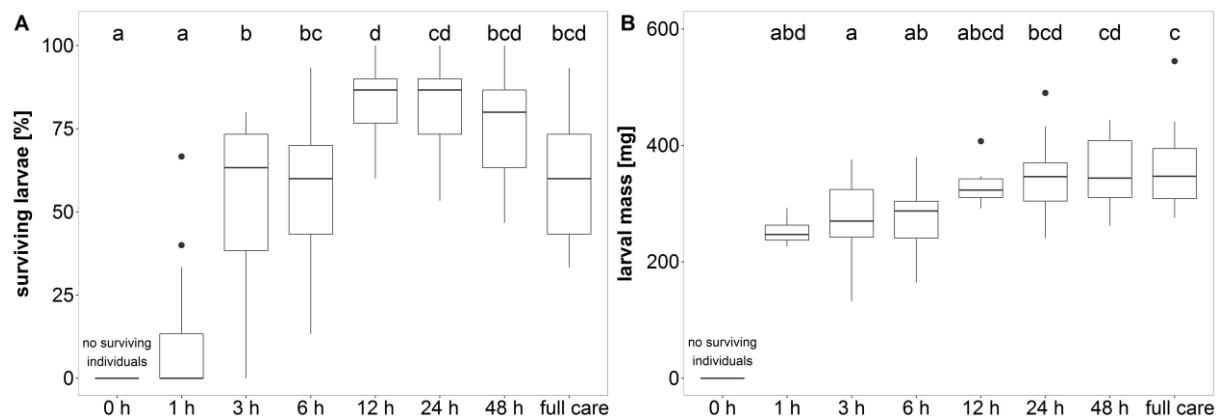
783 **Fig. 4**



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786 **Fig. 5**



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