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(Article begins on next page)

1 *Zerynthia polyxena* locally monophagous on *Aristolochia pallida* in the Susa Valley

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7
8 **Abstract**

9 In insect-plant biology, oviposition choices and larval development on different host plants are crucial
10 factors to be investigated. To design conservation strategies for protected Lepidoptera, which are
11 overall oligophagous but locally monophagous, it is important to understand which host plant species
12 is locally preferred. We thus investigated oviposition choices and larval development of the protected
13 butterfly *Zerynthia polyxena* in controlled laboratory conditions, using three possible host plant
14 species which are present in the Piedmont region: *Aristolochia pallida*, *A. clematitidis* and *A. rotunda*.
15 We found that laboratory conditions are not favorable for *Z. polyxena* oviposition, even if the fertility
16 of the females was in normal range for Papilionidae. However, we were able to understand the local
17 monophagy of the species on *A. pallida* in the Susa Valley through larval survival and development
18 stages. Egg hatching was similar among the three host plant species; however, even if larvae eat and
19 grow similarly on the different host plant species until the third larval stage, the only larvae that
20 reached the pupal stage were those fed with *A. pallida*.

21 In conclusion, whereas *Z. polyxena* is oligophagous in the rest of Europe for the genus *Aristolochia*,
22 the species is locally monophagous on *A. pallida* in the Susa Valley.

23
24 Keywords: oviposition, larval host plant, larval growth, butterflies, laboratory rearing.

25
26 **Introduction**

27 Oviposition preference and larval performance on different host plants are central topics in insect-
28 plant biology (Renwick and Chew 1994, Xue et al. 2007, García-Barros and Fartmann 2009). Indeed,
29 for most herbivorous insects, first larval instars do not switch the host plant on which they were
30 hatched. Thus oviposition, and in particular host plant selection, is a crucial factor for future
31 development of larvae (e.g. Gripenberg et al. 2010). The host plant choice might be related to genetic
32 adaptation to the local host plant species (e.g. Kuussaari et al. 2000) or to Hopkins' host selection
33 principle (Hopkins 1917), for which females lay eggs on the host plant species where they were
34 hatched (Ning et al. 2018). Moreover, for the *performance hypothesis*, host plant species selection is
35 linked to larval growing performance (e.g. Cini et al. 2019), which might also be linked to host plant
36 local abundance, phenology, nutritional quality and chemical defenses (e.g. Awmack and Leather
37 2002). Some oligophagous Lepidoptera species can be locally monophagous (e.g. Meister et al. 2015).
38 For these species, host plant distribution is a key factor in shaping butterfly spatial distribution (e.g.
39 Čelik 2012) and, therefore, oviposition choice and larval development on different host species are
40 crucial aspects that require investigations for conservation purposes.

41 The southern festoon, *Zerynthia polyxena*, is a Papilionidae butterfly protected in the EU (Habitats
42 Directive 92/43/EEC) and it is currently declining in Europe and Italy (evaluated together with *Z.*
43 *cassandra* before 2013: Van Swaay et al. 2010, Bonelli et al. 2011. *Z. polyxena*, evaluated separately
44 from *Z. cassandra* was estimated Least Concern in the Italian Red List Bonelli et al. 2018. It is a
45 univoltine butterfly that can be found from Central and Southern Europe to the Balkans and Asia
46 Minor. Like other *Zerynthia* species, it is considered oligophagous on *Aristolochia* spp. Middleton-
47 Welling et al. 2020, but it is recorded as locally monophagous (Table 1; *Z. polyxena*, Batáry et al.
48 2008, Čelik 2012; *Z. cerisy*, Slancarova et al. 2015; *Z. cassandra*, Vovlas et al. 2014, Camerini et al.
49 2018, Ghesini et al. 2018, Cini et al. 2019. Understanding if the species are locally monophagous is
50 crucial in order to detect vulnerable species and anticipate a possible mismatch between butterfly-
51 plant phenology (e.g. Cerrato et al. 2016). If this is the case, *ad hoc* conservation strategies need to
52 be planned accordingly.

53

54 Table 1: *Zerynthia* species, their host plant species and the locality. The definitions of the type of
55 phagism in the second column follow Middleton-Welling et al. 2020.

56

57 Because more than one *Aristolochia* spp. are present in Italy, the aim of this study is to
58 investigate if *Z. polyxena* is locally monophagous in the Susa Valley by examining:

- 59
- *Z. polyxena* oviposition choices on different host plants;
 - the influence of different host plant species on survival, larval growth and feeding rates,
61 through controlled laboratory rearing.
- 62

63 **Materials and methods**

64 In the Piedmont region of Italy, there are three *Aristolochia* species (*A. pallida*, *A. clematitis* and *A.*
65 *rotunda*) and two *Zerynthia* species (*Z. polyxena* and *Z. cassandra*) (Dapporto 2010, Zinetti et al.
66 2013, Fig. 1). In the study area (place 45°07'47.3"N 6°59'16.0"E) a large *Z. polyxena* metapopulation
67 occurs and both *A. pallida* and *A. clematitis* are present. All these *Aristolochia* species are known to
68 be host plants of *Zerynthia* species in Europe (Örvössy et al. 2005, Vovlas et al. 2014, Camerini et
69 al. 2018).

70

71 Fig. 1: Distribution map of *Aristolochia* species and *Zerynthia polyxena* and *Z. cassandra* in the
72 Piedmont region (research verified data from iNaturalist confirming *Aristolochia* distribution by
73 Nardi 1984).

74

75 **Oviposition experimental design**

76 The oviposition choices of *Z. polyxena* were investigated by conducting both no-choice (only one
77 host plant species at a time) and multi-choice (all three species together) tests.

78 *Aristolochia* plants were collected in the field and three plants of the same species were
79 planted in each pot. Pots were also prepared with flowers, specifically *Viola* and *Muscari* species
80 flowering since the middle of March. About 60 pots in total were prepared. To avoid the possibility
81 that secondary metabolites produced as a consequence of replantation might affect oviposition, each
82 pot was chilled for at least 48 hours before being used for experiments.

83 **No-choice preference tests** were performed by placing three pots of the same species in a
84 cage (1m × 1m × 1m) where 5 females and a variable number of males (Table A1 in Appendix), all
85 collected in the field during the same morning, were introduced. The adults were left in the cage for
86 24 hours, after which they were removed and the eggs that they deposited were counted. The same
87 preference test was performed for all the plant species, with different individuals and different pots,
88 and we replicated the test 3 times per species (for a total of 9 tests).

89 **Multi-choice preference tests** were performed by placing three potted plants, one *A.*
90 *clematitis*, one *A. rotunda* and one *A. pallida* in a cage (1m × 1m × 1m) where 5 females and a variable
91 number of males (Table A1), all collected in the field during the same morning, were introduced. All
92 the plants selected for the experiment showed (had) a similar amount of leaves. The test was
93 performed 6 times with different adults and different pots. The adults were left in the cage for 24
94 hours, after which they were removed and the eggs that they deposited were counted.

95 Males were added in the preference tests because in an experimental trial a mating was
96 recorded (conversely to what was observed by Cini et al. 2019). Cages were placed on the table at the
97 windows south-facing in the laboratory and half of the cages were exposed to direct sunlight during
98 the central hours of the day. At the end of the experiments, all the adults were released in the same
99 location where they were collected.

100 The butterflies that did not survive the oviposition tests were replaced with new ones. In order
101 to investigate the **fertility** of the dead butterflies, all the abdomens were dissected and the number of
102 mature eggs counted, which are easily identified thanks to their yellow color and bigger size

103 compared to the non-mature ones which appear whitish and small (Watanabe et al. 1986). The size
104 of the mature eggs and the presence of spermatheca were also recorded.

105 To understand oviposition preferences, other experimental trials were conducted but brought
106 no significant results and thus are not presented here (see details in the Appendix).

107

108 **Larval rearing experiments**

109 From April 15th, 2019 to May 15th, 2019 (the oviposition period of the species), 78 eggs
110 belonging to 13 clusters in the field were collected. In the laboratory, each egg was carefully removed
111 from the deposition leaf and then moved to new leaves, equally dividing the eggs among the three
112 *Aristolochia spp.* under investigation (26 eggs per species; Table A2 in Appendix). When possible,
113 eggs belonging to the same clusters were equally divided among all three possible host plant species.
114 Each egg with its leaf was placed singularly in a 5 cm diameter Petri dish (according to Jordano and
115 Gomariz 1994) and its development from hatching until pupation was recorded. After the egg
116 hatched, each leaf that housed the larvae was weighed and the petiole of the leaf was placed inside
117 moist cotton to maintain its turgor (Cini et al. 2019). Each Petri dish was placed in a climatic chamber
118 (Memmert HPP260) where both temperature and humidity were kept constant at 20°C and 60%
119 respectively, with a 12L:12D photo-period. The temperature and humidity levels selected correspond
120 to an average recorded during the 10 days prior to the start of the experiment.

121 Each Petri dish was checked every day to control the larvae growth, the possible change of
122 instar (evaluated by the presence of exuvia) and the leaf turgor. Unconsumed leaf materials and new
123 leaves were weighed and changed every 2 days. In addition, the larvae were measured (weight and
124 length) as they hatched and each time they changed larval instar. From the third instar onwards, they
125 were measured every two days.

126 Following Cini et al. (2019), when larvae were close to pupation, recognizable from their reduced
127 mobility and food intake, they were moved to a plastic box together with a stem of the host plant and
128 a wood stick in order to facilitate pupation. Once the pupation process was completed, the pupae were
129 measured and then left in the box in an unheated room until the next season. The next spring, the
130 butterflies that emerged from those pupae were released in the same area where the eggs were
131 collected.

132

133 **Statistical analysis**

134 To understand if hatching might depend on the host plant species where the egg was placed,
135 hatched (1) and not-hatched (0) eggs were modelled using a generalized linear model (GLM,
136 Binomial, maximum likelihood fit) where host plant species were used as additive categorical
137 explanatory variables. Egg clusters were added as random intercept term.

138 To understand if larval survival depends on host plants, non parametric Kaplan-Meier method
139 in R survival package was applied using life length (in days) as response variable.. Then we used Cox
140 proportional-hazard regression.

141 Larval lengths and weights were positively correlated (Pearson correlation = 0.93, $p < 0.001$).
142 Given this correlation, larval lengths were modelled in a generalized linear mixed model (GLMM,
143 Gamma, maximum likelihood fit) where host plant species were added as an additive explanatory
144 variable. To adjust for multiple observations of the same larvae, it was decided to include larval code
145 as a random intercept and measurement day as a random slope term.

146 To understand if larval feeding depends on the host plant species, we modelled subtractions
147 of initial minus final leaf weights divided by days in which the leaf was left in the Petri in a
148 generalized linear mixed model (GLMM, Gamma, maximum likelihood fit) where the host plant
149 species was added as additive explanatory variable. To adjust for multiple observations of the same

150 larvae, and eating differences related to the larval stage, larval code and stage were included as a
151 random intercept.

152 This model was fitted using the ‘stats’ package in the R (v3.2.1) statistical and programming
153 environment (R Core Team 2005). For all the models *A. clematitidis* was used as reference category.

154

155 **Results**

156 The results showed great difficulty in carrying out oviposition experiments in a laboratory
157 environment. Among 15 tests with a total of 75 *Z. polyxena* females, a cluster of 4 eggs was laid
158 during only one no-choice oviposition test. Of the 75 females, 26 died from unidentified causes
159 without laying eggs. From the dissection of their abdomens, a mean of 42 ± 28 eggs (min=13;
160 max=100) of about 0.70/0.80 mm length (min=0.60/0.70mm; max=0.80/0.90mm; Fig. A1 in
161 Appendix) was found. Moreover, 31% of those abdomens included a full spermatheca, in 42% the
162 spermatheca was empty, and in 27% was uncertain.

163 Even if different amount of eggs hatched on different host plants (24, 19 and 22 eggs hatched
164 respectively on *A. pallida*, *A. clematitidis* and *A. rotunda*.), the analysis found that hatching is not
165 dependent on the host plant species where eggs (N=78) were placed on (*A. pallida*: N=26, Z=1.89,
166 $p=0.059$; *A. rotunda*: N=26, Z=1.30, $p=0.193$). However, the data shows that larval lifespans do
167 depend on feeding plant species (Fig. 2a; mean lifespan on *A. clematitidis* 2,5 days, on *A. pallida* 13,0
168 days and *A. rotunda* 6,3 days; Fig. 2b; Kaplan–Meier test Chi square = 15.1, d.f. = 2, $p < 0.001^{***}$).
169 From Cox regression analysis, the survival was significantly higher on *A. pallida* and *A. rotunda* than
170 *A. clematitidis* (*A. pallida*: Z= -3.75, $p < 0.001^{***}$; *A. rotunda*: Z= -2.28, $p=0.022^*$), but only larvae
171 reared on *A. pallida* reached pupal stage (N 2 larvae). The larval median life span differed when
172 reared on the three species: *A. clematitidis* 2 days; *A. pallida* 9,5 days and *A. rotunda* 3 days.

173

174 Fig. 2: Results on larval life length on different host plant species. a) Egg hatching and larval
175 development as a function over time and b) results from Kaplan–Meier method on larval life length
176 on three different host plant species. In a) only two larvae reached the pupal stage when reared on *A.*
177 *pallida*.

178

179

180 Larval growth was similar until the third larval stage (Fig. 3a), but starting from the fourth
181 stage most of the larvae survive only on *A. pallida*. There were not any significant differences among
182 lengths (N=120 measures) between larvae fed on different host plant species (Fig. 3b; *A. pallida*: t
183 value=-0.75, p=0.454; *A. rotunda*: t value=-0.77, p=0.441). Moreover, no differences were detected
184 on the feeding rate (N=169 leaves) among larvae reared on different host plant species (Fig. A2 in
185 Appendix; *A. pallida*: t value=0.96, p=0.338; *A. rotunda*: t value=1.16, p=0.245).

186

187 Fig. 3: Results on the sizes of larvae growing on different host plant species. a) Larval length along
188 as a function over time and b) Boxplots of larval length divided for each larval stage and host plant
189 species.

190

191 Discussion

192 This study investigated adult oviposition choice, larval growth, and survival of *Z. polyxena*
193 under controlled laboratory conditions. From the oviposition choice tests, it was not possible to assess
194 a preference of the adult towards one of the three plant species. Indeed, females laid eggs (1 cluster
195 with 4 eggs) in only one test out of a total of 15. To understand if avoidance of oviposition was linked
196 to intrinsic fertility of those *Z. polyxena* females, eggs within the abdomens of those females that died
197 during the oviposition tests (26 out of 75 - 34%) were counted. This analysis showed that the mean
198 number and size of mature eggs (42 ± 28 mature eggs and 0.70/0.80 mm egg diameter length) was

199 consistent with other Papilionidae species (see Watanabe et al. 1986). Moreover, a sperm-full
200 spermatheca was recorded in 31% of those females (Fig. A1 in Appendix). A possible explanation
201 for the lack of oviposition could be the fact that laboratory conditions might be not ideal for *Z.*
202 *polyxena*. Even if the cages were larger than those typically used (e.g. Quacchia et al. 2008, Näsvall
203 et al. 2021), and light and temperature were maintained in accordance with field base conditions,
204 females did not lay eggs (with one exception). Cini et al. (2019) experienced similar egg-laying
205 avoidance for *Z. cassandra*. Even if these failures are rarely reported in scientific papers, some
206 research confirmed rearing difficulties and high mortality among adult butterflies (e.g. Herms et al.
207 2017, Näsvall et al. 2021).

208 Despite the challenges cited above, it is possible to infer the plant preference by looking at
209 the results of the larval development. Local monophagy might be driven by larval performance (in
210 accordance with Wiklund et al. 2018). Contrary to other parts of Europe where *Z. polyxena* is
211 oligophagous or monophagous on *A. clematitis* (Batáry et al. 2008), laboratory rearing showed that
212 larvae have a higher rate of survival on *A. pallida*. For *Z. Cassandra* in central Italy, the association
213 with host plants is not so strong, even if *adults* that were reared on *A. rotunda* before pupation, showed
214 a longer life-span (Cini et al. 2019). In this study, six larvae (25% of larvae) reached the fifth instar
215 of which only two larvae reached the pupal stage and all of them were reared on *A. pallida*. This
216 might be related to the phenological status of the host plant that affects oviposition (Patricelli et al.
217 2011). It has already recorded for *Maculinea* (=Phengaris) *arion* that changes oviposition preferences
218 in relation the host plant species phenological status: females that fly during spring lay eggs
219 on *Thymus spp.*, while those that fly during summer prefer to lay eggs on *Origanum vulgare*
220 (Berezki et al. 2014, Osváth-Ferencz et al. 2017). Even for *Euphydryas matura* larvae, host plant
221 phenology might be a key factor. This butterfly usually lays eggs on *Fraxinus excelsior* (except in
222 Finland), but larvae might change host plant after hibernation due to host plant availability – as
223 occurs in Austria – or to phenology – as occurs in Italy (Dolek et al. 2013). In this study area, *A.*
224 *pallida* has a different phenological status compared with *A. clematitis* which develops and is

225 abundant only at the end of the flight period of *Z. polyxena* adults (personal observation). This
226 suggests that, due to a phenological mismatch between the adults' flight period and host plant
227 phenology, the population in the north of Italy has undergone local adaptation in response to the local
228 abundance of *A. pallida* (Janz et al. 2001). Indeed, it has already proven for *Zerynthia cassandra* that
229 the host plant phenology has a strong effect on oviposition preferences (for example presence of
230 flowers is positively correlated with the abundance of eggs; e.g. Cini et al. 2019).

231 Furthermore, since different species of *Aristolochia* differ in the amount of aristolochic acids
232 they contain (see Cateni et al. 1993), *Z. polyxena* in the Susa Valley might have developed
233 characteristics that lead the population to better tolerate the specific amount of aristolochic acids
234 present in *A. pallida*, rather than in *A. clematitis* or *A. rotunda*. As for the latter, even if it has a similar
235 phenology to *A. pallida*, it is not abundant in the North West of the Piedmont region. This might
236 explain why larvae reared *A. clematitis* avoided to eat while those reared on *A. rotunda* do not reach
237 the fourth instar (except for one that died after few days). Indeed, the accumulation of different
238 amount of aristolochic acids might be tolerated until a certain level. Larvae have similar growth in
239 terms of length and weight and have eaten a similar amount of leaves when reared on *A. pallida* and
240 *A. rotunda* (Fig. A2 in Appendix) but most of them do not reach the fourth instar. Moreover, some
241 species might be more tolerant than others. Indeed, *Battus philenor* does not experience different
242 larval survival rates when fed on *Aristolochia californica* or on the same species with supplemented
243 aristolochic acids (Fordyce 2001). As a caveat, different larval length and weight did not take into
244 account that females might be larger than males (Cini et al. 2019).

245 In conclusion, *Z. polyxena* in Susa Valley is locally monophagous on *A. pallida*, contrary to what has
246 been observed in the rest of the EU, where normally *A. clematitis* is preferred (Batáry et al. 2008).
247 These two *Aristolochia* species, even if sympatric in most parts of Italy, show different microhabitat
248 preference in terms of tree cover, light exposition, and elevation range (Pignatti et al. 2017). Given
249 the strong association between insect and host plant, especially for locally monophagous species, this
250 result is particularly important for conservation purposes. Indeed, *Z. polyxena* distribution and

251 abundance is strongly linked to the presence of host plants and micro-habitat conditions such as sun
252 exposure (e.g. Čelik 2012, Cini et al. 2021), that might be change in relation to host plant species.
253 Moreover, for a successful conservation plan that included a host plant transplantation (as already
254 carried out in the province of Bologna by Ghesini et al. 2018) it is crucial to know exactly which host
255 plant species is locally used by *Z. polyxena*.

256

257

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267

268 SB and IP conceived the ideas and designed methodology; IP and VDP collected the data; IP analysed
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270 and gave final approval for publication.

271

272

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404 Fig. 1: Distribution map of *Aristolochia* species and *Zerynthia polyxena* and *Z. cassandra* in the
405 Piedmont region (research verified data from iNaturalist confirming *Aristolochia* distribution by
406 Nardi 1984).

407

408 Fig. 2: Results on larval life length on different host plant species. a) Egg hatching and larval
409 development as a function over time and b) results from Kaplan–Meier method on larval life length
410 on three different host plant species. In a) only two larvae reached the pupal stage when reared on *A.*
411 *pallida*.

412

413 Fig. 3: Results on the sizes of larvae growing on different host plant species. a) Larval length along
414 as a function over time and b) Boxplots of larval length divided for each larval stage and host plant
415 species.

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417 Table 1: *Zerynthia* species, their host plant species and the locality. Narrow oligophagous definition
418 follows Middleton-Welling et al. 2020.

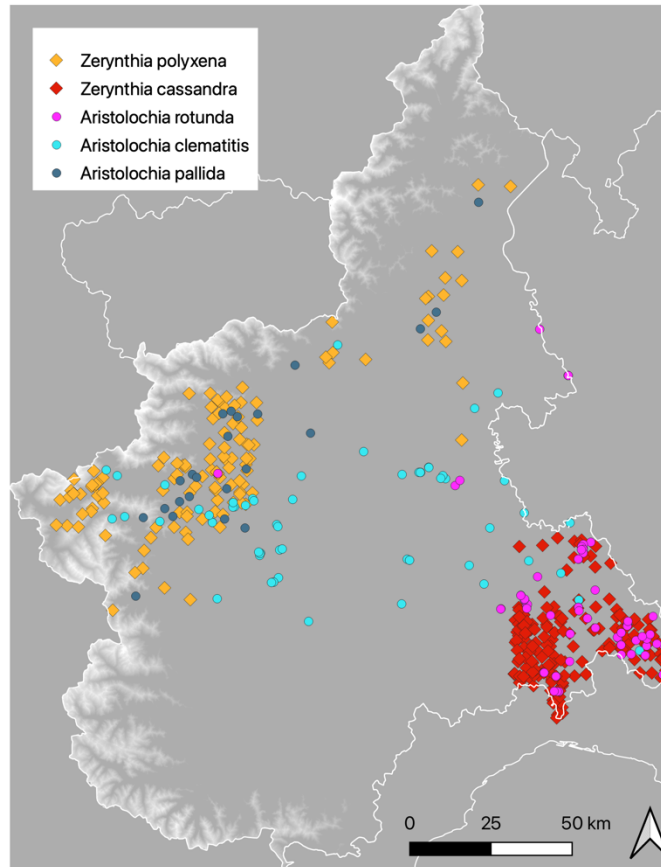
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Butterfly species	Phagism	Host plant species	Region	Reference
<i>Z. polyxena</i>	Narrow oligophagous	<i>A. lutea</i> , <i>A. clematitidis</i> , <i>A. pallida</i>	Slovenia	Špiljar 2007, Verovnik et al. 2009
	Locally monophagous	<i>A. clematitidis</i>	SE Slovenia	Čelik 2010, 2010b
	Locally monophagous	<i>A. lutea</i>	SW Slovenia	Čelik 2012
	Locally monophagous	<i>A. clematitidis</i>	Central Hungary	Batáry et al. 2008
	Narrow oligophagous	<i>A. pallida</i> , <i>A. rotunda</i> , <i>A. clematitidis</i>	Greek Thrace	Slancarova et al. 2015
	Locally monophagous	<i>A. pallida</i>	Piedmont (Italy)	This study
<i>Z. cerisy</i>	Narrow oligophagous	<i>A. clematitidis</i> , <i>A. hirta</i> , <i>A. rotunda</i>	Greek Thrace	Slancarova et al. 2015
<i>Z. cassandra</i>	Narrow oligophagous	<i>A. lutea</i> , <i>A. rotunda</i>	Elba island (Italy)	Cini et al. 2019, Cini et al. 2021
	Locally monophagous	<i>A. rotunda</i>	Piedmont, Pulia, Emilia-Romagna and Lombardia (Italy)	Vovlas et al. 2014, Camerini et al. 2018, Ghesini et al. 2018, Ghesini et al. 2019;

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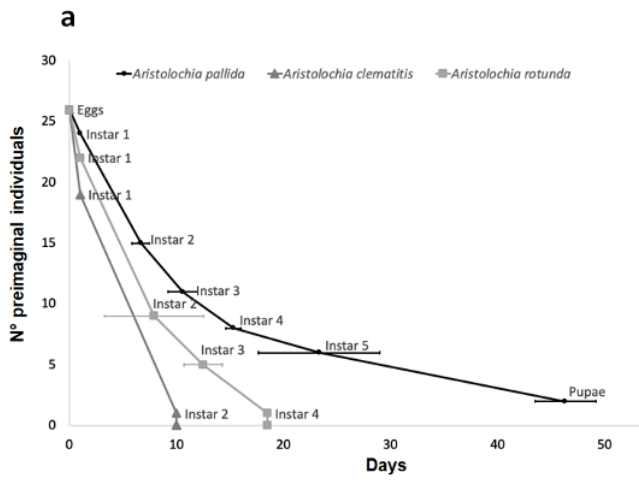
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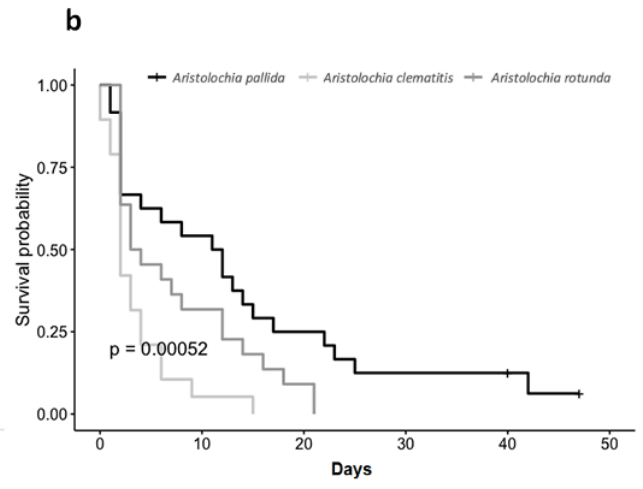
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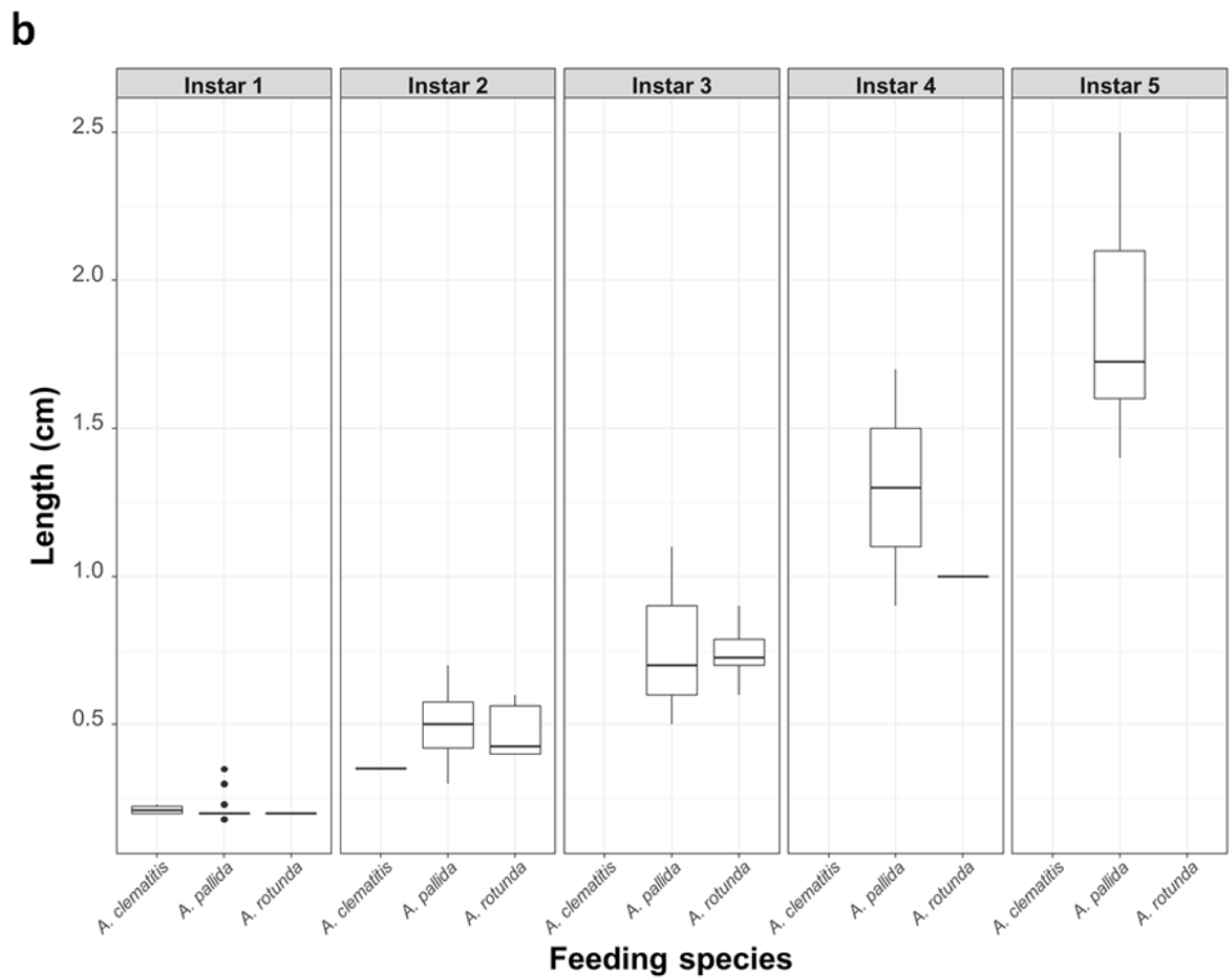
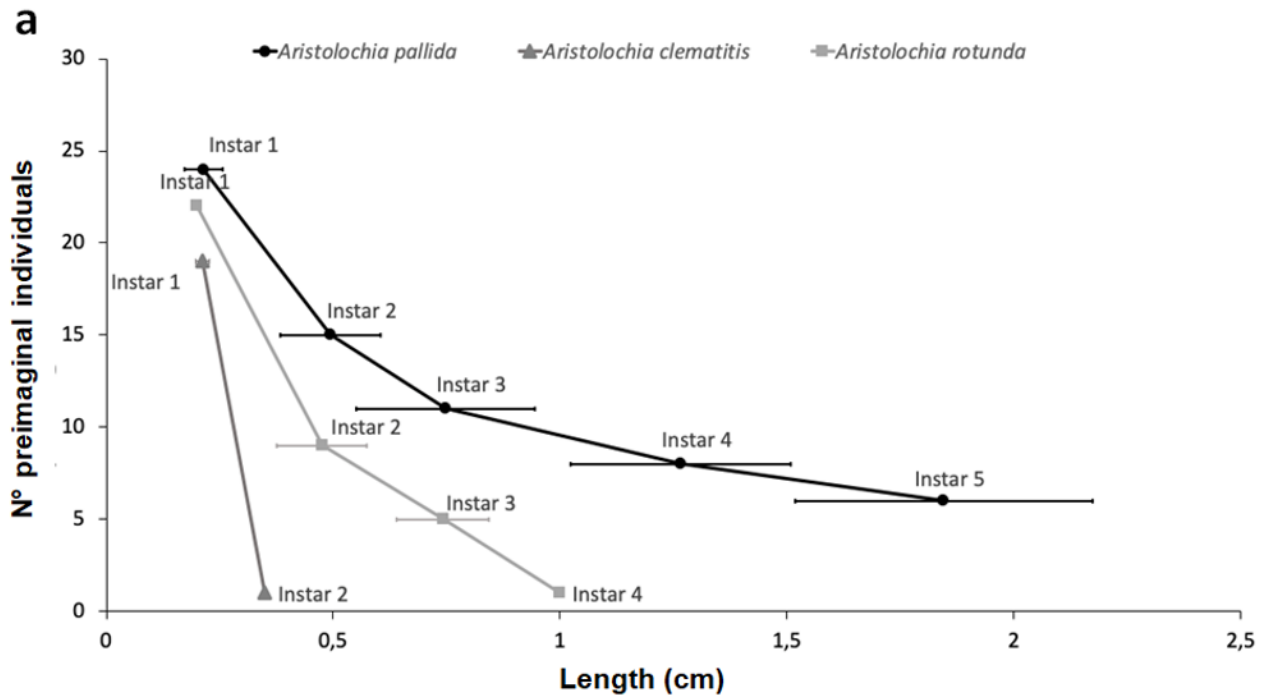
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