Zerynthia polyxena Locally Monophagous on Aristolochia pallida in the Susa Valley

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

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Abstract

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

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

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

Introduction

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

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

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

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

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

Materials and methods

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

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

<u>Oviposition experimental design</u>

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

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

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

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

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

The butterflies that did not survive the oviposition tests were replaced with new ones. In order to investigate the **fertility** of the dead butterflies, all the abdomens were dissected and the number of mature eggs counted, which are easily identified thanks to their yellow color and bigger size compared to the non-mature ones which appear whitish and small (Watanabe et al. 1986). The size of the mature eggs and the presence of spermatheca were also recorded.

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

Larval rearing experiments

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

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

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

Statistical analysis

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

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

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

To understand if larval feeding depends on the host plant species, we modelled subtractions of initial minus final leaf weights divided by days in which the leaf was left in the Petri in a generalized linear mixed model (GLMM, Gamma, maximum likelihood fit) where the host plant species was added as additive explanatory variable. To adjust for multiple observations of the same larvae, and eating differences related to the larval stage, larval code and stage were included as a random intercept.

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

Results

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

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

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

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

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

Discussion

This study investigated adult oviposition choice, larval growth, and survival of *Z. polyxena* under controlled laboratory conditions. From the oviposition choice tests, it was not possible to assess a preference of the adult towards one of the three plant species. Indeed, females laid eggs (1 cluster with 4 eggs) in only one test out of a total of 15. To understand if avoidance of oviposition was linked to intrinsic fertility of those *Z. polyxena* females, eggs within the abdomens of those females that died during the oviposition tests (26 out of 75 - 34%) were counted. This analysis showed that the mean number and size of mature eggs (42±28 mature eggs and 0.70/0.80 mm egg diameter length) was consistent with other Papilionidae species (see Watanabe et al. 1986). Moreover, a sperm-full spermatheca was recorded in 31% of those females (Fig. A1 in Appendix). A possible explanation for the lack of oviposition could be the fact that laboratory conditions might be not ideal for *Z. polyxena.* Even if the cages were larger than those typically used (e.g. Quacchia et al. 2008, Näsvall et al. 2021), and light and temperature were maintained in accordance with field base conditions, females did not lay eggs (with one exception). Cini et al. (2019) experienced similar egg-laying avoidance for *Z. cassandra*. Even if these failures are rarely reported in scientific papers, some research confirmed rearing difficulties and high mortality among adult butterflies (e.g. Herms et al. 2017, Näsvall et al. 2021).

Despite the challenges cited above, it is possible to infer the plant preference by looking at the results of the larval development. Local monophagy might be driven by larval performance (in accordance with Wiklund et al. 2018). Contrary to other parts of Europe where *Z. polyxena* is oligophagous or monophagous on *A. clematitis* (Batáry et al. 2008)*,* laboratory rearing showed that larvae have a higher rate of survival on *A. pallida*. For *Z. Cassandra* in central Italy*,* the association with host plants is not so strong, even if *adults* that were reared on *A. rotunda* before pupation, showed a longer life-span (Cini et al. 2019). In this study, six larvae (25% of larvae) reached the fifth instar of which only two larvae reached the pupal stage and all of them were reared on *A. pallida*. This might be related to the phenological status of the host plant that affects oviposition (Patricelli et al. 2011). It has already recorded for *Maculinea* (*=Phengaris*) *arion* that changes oviposition preferences in relation the host plant species phenological status: females that fly during spring lay eggs on *Thymus spp.,* while those that fly during summer prefer to lay eggs on *Origanum vulgare* (Bereczki et al. 2014, Osváth-Ferencz et al. 2017). Even for *Euphydryas matura* larvae, host plant phenology might be a key factor. This butterfly usually lays eggs on *Fraxinus excelsior* (except in Finland), but larvae might change host plant after hibernation due to host plant availability – as occurs in Austria – or to phenology – as occurs in Italy (Dolek et al. 2013). In this study area, *A. pallida* has a different phenological status compared with *A. clematitis* which develops and is abundant only at the end of the flight period of *Z. polyxena* adults (personal observation). This suggests that, due to a phenological mismatch between the adults' flight period and host plant phenology, the population in the north of Italy has undergone local adaptation in response to the local abundance of *A. pallida* (Janz et al. 2001). Indeed, it has already proven for *Zerynthia cassandra* that the host plant phenology has a strong effect on oviposition preferences (for example presence of flowers is positively correlated with the abundance of eggs; e.g. Cini et al. 2019).

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

In conclusion, *Z. polyxena* in Susa Valley is locally monophagous on *A. pallida*, contrary to what has been observed in the rest of the EU, where normally *A. clematitis* is preferred (Batáry et al. 2008). These two *Aristolochia* species, even if sympatric in most parts of Italy, show different microhabitat preference in terms of tree cover, light exposition, and elevation range (Pignatti et al. 2017). Given the strong association between insect and host plant, especially for locally monophagous species, this result is particularly important for conservation purposes. Indeed, *Z. polyxena* distribution and abundance is strongly linked to the presence of host plants and micro-habitat conditions such as sun exposure (e.g. Čelik 2012, Cini et al. 2021), that might be change in relation to host plant species. Moreover, for a successful conservation plan that included a host plant transplantation (as already carried out in the province of Bologna by Ghesini et al. 2018) it is crucial to know exactly which host plant species is locally used by *Z. polyxena*.

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SB and IP conceived the ideas and designed methodology; IP and VDP collected the data; IP analysed the data; IP and VDP led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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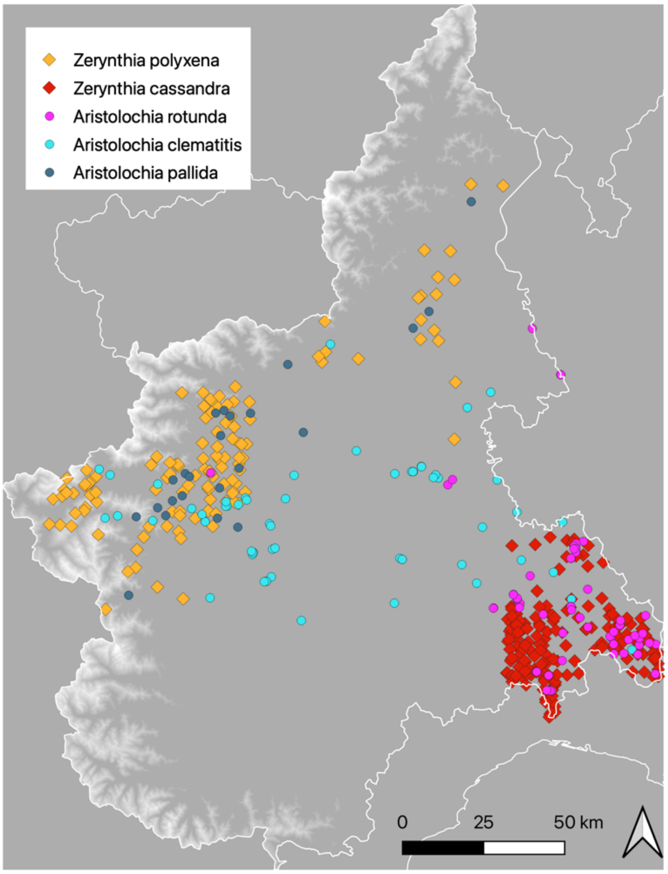
Fig. 1: Distribution map of *Aristolochia* species and *Zerynthia polyxena* and *Z. cassandra* in the Piedmont region (research verified data from iNaturalist confirming *Aristolochia* distribution by Nardi 1984).

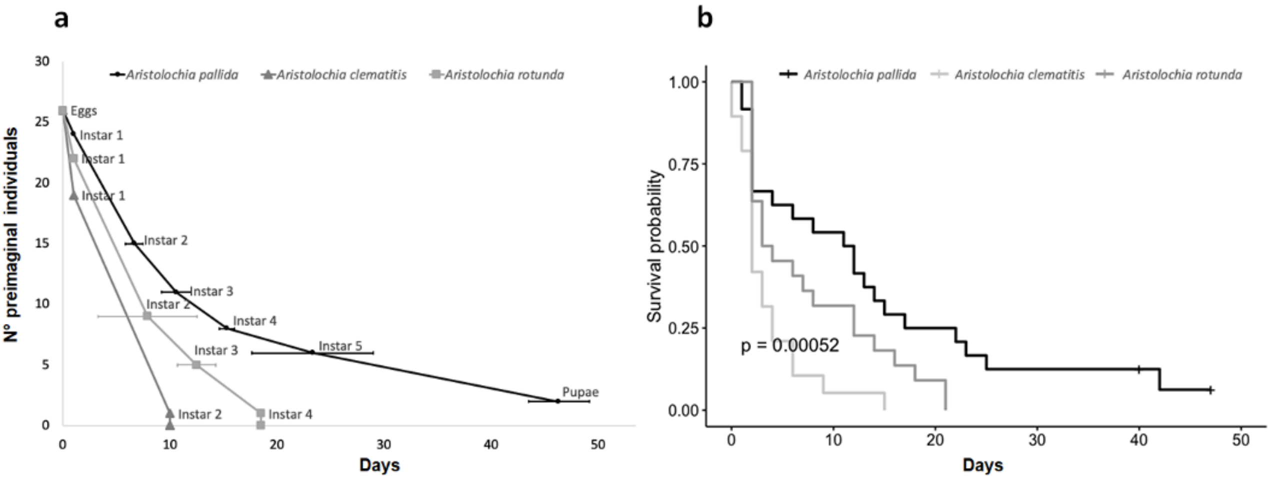
Fig. 2: Results on larval life length on different host plant species. a) Egg hatching and larval development as a function over time and b) results from Kaplan–Meier method on larval life length on three different host plant species. In a) only two larvae reached the pupal stage when reared on *A. pallida*.

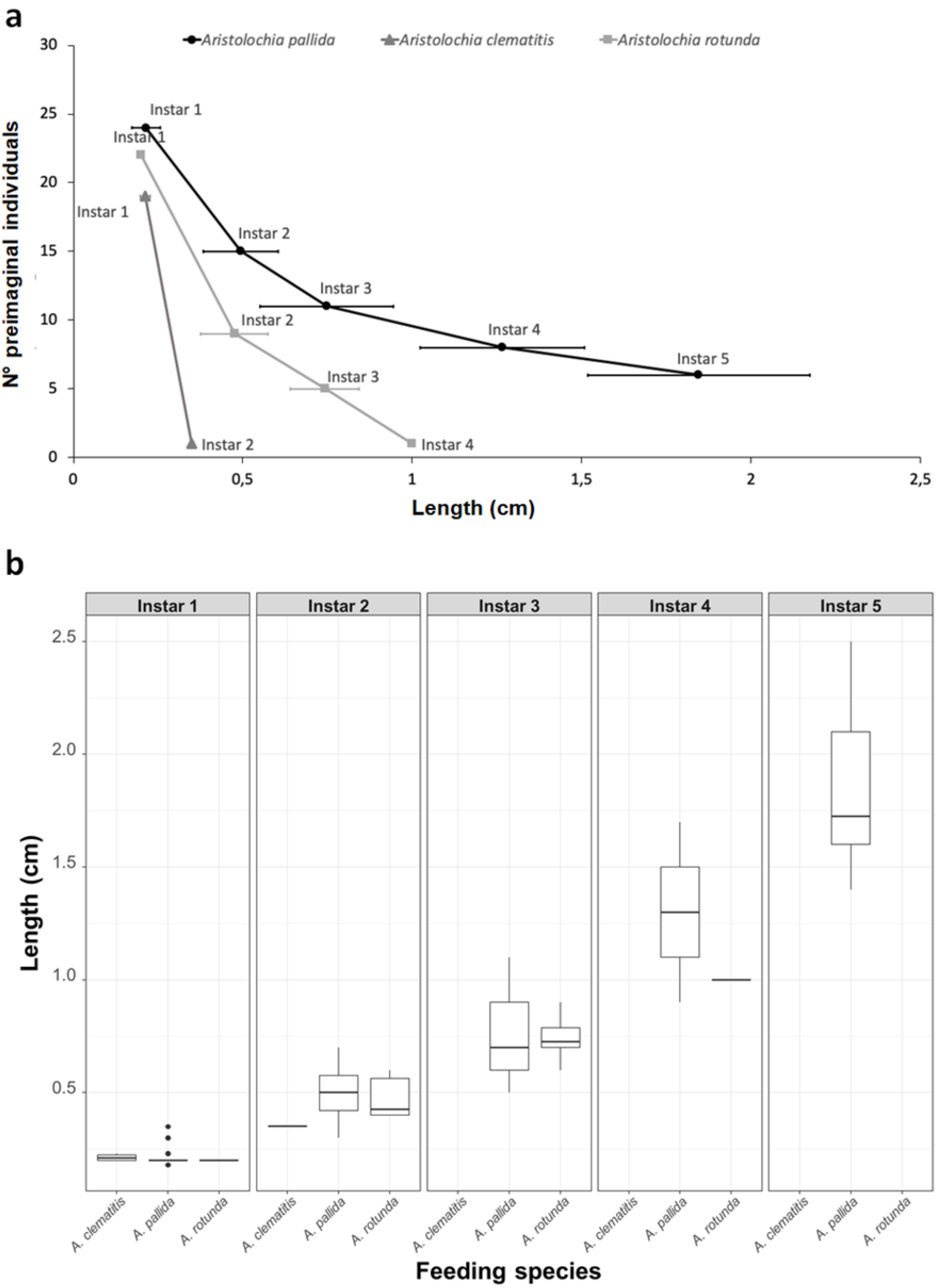
Fig. 3: Results on the sizes of larvae growing on different host plant species. a) Larval length along as a function over time and b) Boxplots of larval length divided for each larval stage and host plant species.

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

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


◆ Zerynthia polyxena
◆ Zerynthia cassandra
● Aristolochia rotunda
● Aristolochia clematitis
● Aristolochia pallida
0
25
50 km


a
N° preimaginal individuals
Days
Aristolochia pallida
Aristolochia clematitis
Aristolochia rotunda
Eggs
Instar 1
Instar 1
Instar 1
Instar 2
Instar 3
Instar 2
Instar 4
Instar 3
Instar 5
Pupae
Instar 2
Instar 4
30
25
20
15
10
5
0
0
10
20
30
40
50
b
Survival probability
Days
Aristolochia pallida
Aristolochia clematitis
Aristolochia rotunda
p = 0.00052
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50


a
Aristolochia pallida
Aristolochia clematitis
Aristolochia rotunda
N° preimaginal individuals
30
25
20
15
10
5
0
Instar 1
Instar 1
Instar 1
Instar 2
Instar 2
Instar 2
Instar 3
Instar 3
Instar 4
Instar 4
Instar 5
0
0,5
1
1,5
2
2,5
Length (cm)
b
Instar 1
Instar 2
Instar 3
Instar 4
Instar 5
Length (cm)
2.5
2.0
1.5
1.0
0.5
A. clematitis
A. pallida
A. rotunda
Feeding species