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Voltinism and larval population structure of *Calopteryx splendens* (Odonata: Calopterygidae) in the Po Valley

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Adaptation of life-history traits is an important factor for the success of insects. Voltinism is a feature that descends from several life-history traits and, given that the latter depend on the specific environment of growth, voltinism can vary between populations across latitudes or habitats. In addition, some insects, like many odonates, have developed different patterns of voltinism within the same population, due to mechanisms of cohort splitting. *Calopteryx splendens* (Odonata: Calopterygidae) is a widespread damselfly in Europe that has been extensively studied regarding its evolutionary ecology, but detailed studies about its voltinism are relatively scarce and confined to the central and northern areas of Europe. So we investigated the voltinism and larval development of a population of *C. splendens* both by captive rearing and in the field in Northern Italy, the southernmost area in which its voltinism has been studied so far. We found an earlier start of larval growth, with respect to previous studies. Additionally, the head-width of larvae of the same instar decreased with the cohort ageing. Finally, the results are consistent with a two-groups emerging pattern that may support partial semivoltinism or partial bivoltinism of *C. splendens* in Northern Italy.

Keywords: damselfly; dragonfly; larvae; life-history; Italy

Introduction

Insects have developed complex life cycles and life-history traits that allowed them to adapt to and colonize different habitats. Additionally, life-history traits have usually evolved to be plastic in response to changing factors (Nylin & Gotthard, 1998), allowing adaptation to spatially and temporally changing environments.

In odonates, age and size at maturity are probably the most relevant life-history traits (Stoks, Johansson, & De Block, 2008). These traits depend mainly on development rate, which in turn is influenced by the biotic and abiotic conditions experienced by larvae in their environment (Atkinson & Sibly, 1997; Blanckenhorn & Demont, 2004; Houston & McNamara, 1992; Johansson, Stoks, Rowe, & De Block, 2001; Nylin & Gotthard, 1998). For example, time constraints imposed by seasonal habitats usually determine a faster development, which leads to a reduction of adult size (Johansson & Rowe, 1999; Johansson et al., 2001; Stoks et al., 2008) during the breeding season.

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Another trait that is strictly related to developmental rate and to several life history traits, and important to adapt to seasonal environments, is voltinism (the number of generations completed in one year). Voltinism can vary between populations within species; generally, populations of a species which occur over a wide latitudinal range tend to show a negative correlation between voltinism and latitude (Braune, Richter, Söndgerath, & Suhling, 2008; Corbet, 1980; Corbet, Suhling, & Soendgerath, 2006; Flenner, Richter, & Suhling, 2010). Voltinism may also depend on variations in larval development rate due to temperature and photoperiod (Corbet et al., 2006), or other biotic and abiotic factors such as larval habitat, food resources and predation risk (Corbet et al., 2006; Martin, Johnson, & Moore, 1991; Stoks et al., 2008). In addition, many odonates have developed different patterns of voltinism within the same population (Corbet et al., 2006; Johansson & Norling, 1994) due to mechanisms of cohort splitting, with some individuals of a hatching cohort that emerge one year sooner or later than the rest of the individuals.

Although the genus *Calopteryx* (Odonata: Calopterygidae) is common in Europe, the majority of studies about the voltinism of its species have been performed in central and northern areas of the continent (reviewed in Corbet et al., 2006). in particular, *Calopteryx splendens* Harris has been shown to generally adopt univoltinism in central Europe (Schütte, Ott, & Hünken, 1999; Schütte & Schrimpf, 2002), although signs of a semivoltine pattern has also been found in one study (Göcking, 1999). Detailed studies on voltinism in this species are scarce, and the larval development pattern is not still clear, due to sampling gaps or low number of sampled individuals. Additionally, this species occurs over a wide latitudinal range and data from southern populations are still lacking. It seems likely that *C. splendens* develops faster in warmer climates with a longer growing season in southern Europe, compared to populations from central and northern Europe. Since this species is widely studied as a model organism in evolutionary ecology, more information about its voltinism is required to interpret correctly findings about *C. splendens* evolutionary and reproductive ecology.

Thus, the aim of this study was to analyse in detail a population of *C. splendens* larvae in Northern Italy, both by periodically sampling one population in the field and by captive rearing, in order to clarify its voltinism and its development in southern Europe.

Materials and methods

Sampling and measurement

Sampling was carried out periodically from 12 March 2014 to 27 July 2015 (see Table 1 for details; the longest period between two subsequent samplings was about a month), in a stream near Pavia (45.16735° N, 9.08762° E; 60 m asl) along a patch of 150 m with uniform vegetated banks, which represent the most common breeding habitat of *C. splendens* in Northern Italy. Mean stream width was 4 m and its depth varied seasonally between 0.6 m and 1.2 m. The site is far from populations of the congeneric *C. virgo*, and this excluded any potential confusion of larval identification.

Larvae were collected with a hand net (mesh size: 0.5 mm) in emergent and submerged vegetation. The vegetation was inserted in the net and the net was shaken quickly, maintaining it parallel to the water flow. After that, debris and larvae harvested in the net were put in white low-rimmed trays. The inside and outside of the net were washed with water inside the trays, in order to collect all the larvae eventually remaining in the net. Finally, larvae were sought and collected visually by accurate inspection of debris and water in the white trays. Samplings lasted 2 hours, with the exception of samplings in which more than 100 individuals were sampled within the first hour, in these cases sampling lasted only one hour (see Table 1 for details). This sampling limitation was due to the need to avoid excessive storage times for individuals during the

Table 1. Summary of dates, number of larvae collected and duration of each sampling of *Calopteryx splendens*.

Date	Number of larvae	Hours sampling
12 March 2014	119	2
25 March 2014	95	2
7 April 2014	112	2
29 April 2014	93	2
13 May 2014	62	2
12 June 2014	80	2
2 July 2014	32	2
17 July 2014	77	2
31 July 2014	130	2
18 August 2014	110	2
4 September 2014	144	2
26 September 2014	144	1
14 October 2014	131	1
28 October 2014	233	2
24 November 2014	111	2
17 December 2014	72	2
19 January 2015	69	2
26 February 2015	21	2
19 March 2015	37	2
24 April 2015	78	2
19 May 2015	86	2
2 July 2015	42	2
23 July 2015	115	1
Total	2193	43

post-sampling manipulation. Additionally, this method allowed standardization of the sampling effort as number of larvae versus sampling hours.

Because individuals below a certain size may not be adequately sampled due to the mesh size of our net (see Lawton, 1970), the frequency of very small larvae might have been underestimated.

After collection, the larvae were stored in water tanks, maintained approximately at stream temperature, and provided with aerators and vegetation as a substrate for the larvae. In the laboratory, the head of each individual was photographed under a microscope (Motic DS-2, Motic, Xiamen, China) and larvae were returned to the sampling site after image collection. The head-width of each individual was measured in three replicates to the nearest 10 μm using the software ImageJ v. 1.48 (Schneider, Rasband, & Eliceiri, 2012), and the mean of the replicates was used as the size of the individual. To investigate the general growth of the larval population, the mean head width of the collected larvae was calculated for each sampling day.

Rearing of larvae

In order to observe the date of emergence of overwintering larvae, 48 larvae with head width ranging from about 1.5 to 2 mm, sampled on 12 March 2014, were kept in the laboratory till emergence, and date of emergence was recorded. Larvae were individually placed inside a net cylinder (7.5 $\times$ 20 cm), and cylinders were placed in tanks (30 $\times$ 36 $\times$ 58 cm) provided with aerators, water pumps and active filters. Tanks were exposed to a photoperiod consistent with that in the field, adjusting light hours, the sunrise and sunset times every week for the entire experiment. Larvae were randomly assigned to two temperature treatments, cold (C: 18°C) and warm (W: 22°C), in order to investigate emergence period at two temperatures, given that temperature is a factor known to affect development rate in odonates, and thus their voltinism (Corbet et al., 2006). We chose 18°C because it is the lower limit for optimal growth in *C. splendens* (Schütte & Schrimpf, 2002; Sternberg & Buchwald, 1999; Zahner, 1959); 22°C was chosen in order to increase temperature, but remaining in the optimum range for the species (Schütte & Schrimpf, 2002; Sternberg & Buchwald, 1999; Zahner, 1959). We did not further increase temperature because this tends to increase oxygen demand, which would have unacceptably reduced the survival of larvae in the laboratory. Larvae were fed every other day with several *Dapnia magna*, *Enchytraeus* spp. and *Chironomus* spp. We verified that the food was enough to last until the next feeding session. The food remained alive between two feeding sessions and was always available to the larvae, lacking possibility of escape from the net cylinder or burial. Uneaten food and waste were removed weekly and half of the water was replaced every month with dechlorinated water.

Results

A total of 2193 larvae were collected in the field (Table 1), photographed and returned to the sampling site. The approximate timing of life history events, such as egg hatching, population growth and emergence of adults, were recognizable from collected data (Figure 1).

Figure 1A shows the number of larvae collected per hour over the entire sampling period. The largest number of individuals was sampled in September/October 2014 and at the end of July 2015. The lowest numbers were caught in July of both years, just before the appearance of the newly hatched small larvae, and between February and March 2015.

The mean head size for each sampling date (Figure 1B) revealed the growth of the population and the appearance of new year classes. The larvae of the first year class grew and presumably emerged up to July 2014, then the larvae of the second year class grew up to July 2015. These data suggest that the majority of the population is univoltine.

In Figure 1C, the two or often three last instars (F-0 to F-2) could be generally discerned as size-frequency peaks, separated from each other and smaller larvae. In particular in the young larvae of the new year-class, sampled from July up to early September, regular instar peaks could also be discerned. Otherwise, during the cold season, peaks in middle sizes were less distinct. In both cases of discernible peaks, the head-width of a particular instar in a year-class decreased in successive samples. In the easily distinguished F-0 and F-1 the decrease in size could be statistically assessed using linear regression analysis (1st year class F-0: $\beta \pm$ standard error (SE) $= -3.38 \pm 0.32$, F $= 112.88$, $p = < 0.0001$; 1st year class F-1: $\beta \pm$ SE $= -2.69 \pm 0.30$, F $= 78.60$, $p = < 0.0001$; 2nd year class F-0: $\beta \pm$ SE $= -2.58 \pm 0.90$, F $= 8.20$, $p = 0.008$; 2nd year class F-1: $\beta \pm$ SE $= -1.48 \pm 0.15$, F $= 95.27$, $p = < 0.0001$). In instars around 1.5 mm, the successive size decrease between mid-July to early September was equal to the normal difference between adjacent instars.

Very small larvae (head width < 0.54 mm) started to appear in the beginning of July both in 2014 and in 2015 (Figure 1C), which indicated hatching. The number of these very small larvae increased till the middle of July 2014, and then decreased. From 18 August to 14 October 2014 they were absent. A further small peak of hatchlings was observed at the end of October.

Larvae in the last instar (F-0) were first observed at the end of March 2014 and peaked near the middle of June (during the period of higher adult density in this population, personal observations). Then, the number of individuals in F-0 decreased to the beginning of July. In the samplings between 2 July and 18 August 2014 no F-0 larvae were observed (Figure 1C). However, at the end of August and in early September few individuals of F-0 were collected. Subsequently, the first F-0 larvae were caught at end of April 2015. In 2015 the highest number of F-0 larvae was found in May and none were found by the end of July, a similar pattern to 2014.

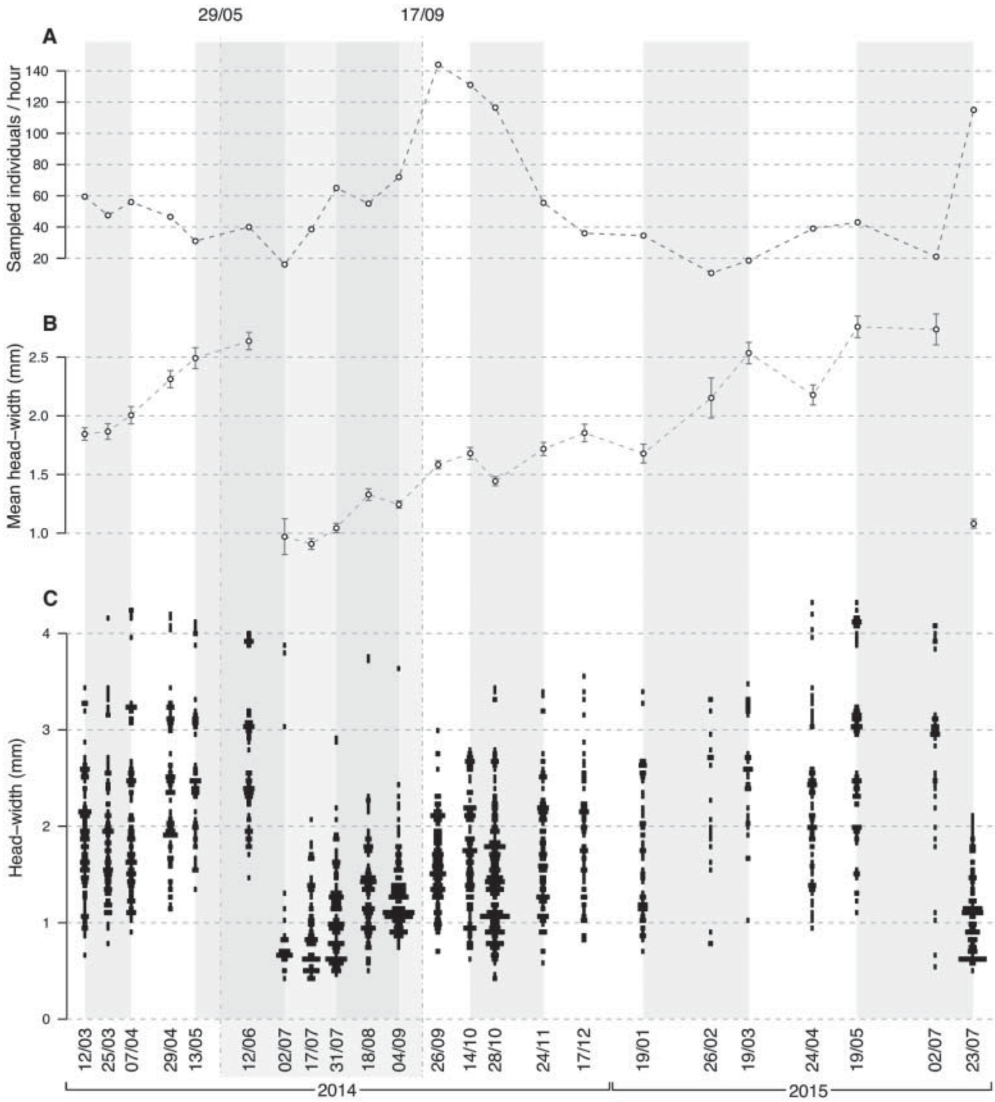

Figure 1. Larval development of *C. splendens* in the field. The grey shade between dot-dash lines, and dates in the upper part of the figure, highlight the period of emergence of adults from the rearing experiment; the darker grey shades help to correlate the graphs with dates. (A) Number of sampled larvae per hour for each sampling in the field. (B) Mean head-width of larvae for each sampling. Bars represent standard error of the mean. Dashed lines approximately indicate the development of the majority of individuals of a year class, those considered univoltine in the text. (C) Kite diagrams showing head-width frequencies in each sampling in the field. The size classes are 0.04 mm.

Rearing experiment

A total of 39 larvae successfully emerged. The first adult emerged on 29 May 2014 (78 days after collection; Figure 2) and the last on 17 September of the same year (189 days after collection; Figure 2). The highest mortality occurred at 22°C with only 17 larvae that completed development (29.2% mortality). Only two larvae out of 24 died prematurely in the 18°C group (8.3% mortality). The number of emerging larvae per week in both temperature treatments peaked between 20 June and 17 July (between 100 and 127 days after collection; Figure 2), the peak in the 22°C treatment (11 larvae in 4 weeks, 64.7% of the total emerging larvae in W) preceded the

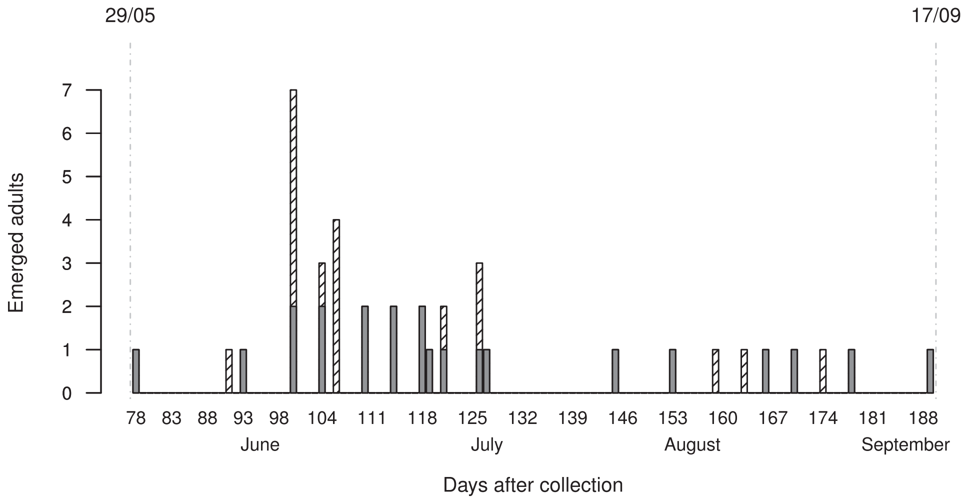

Figure 2. Number of emerged adults in the rearing experiment: grey bars stand for larvae reared at 18°C and shaded bars for larvae reared at 22°C. Dates in the upper part of the figure are those of the first and the last emergence of reared individuals.

peak of the 18°C treatment by about 2 weeks (12 larvae in 4 weeks, 54.6% of the total emerging larvae in C). In both treatments, a small number of adults emerged at the end of August (Figure 2) and no adults emerged between 18 July and 4 August (between 128 and 145 days after collection). This bimodal pattern of emergence is similar to that observed in the field samples.

Discussion

In this study we investigated larval growth and emergence of *C. splendens* in Northern Italy, the southernmost area in which its voltinism has been studied so far (Göcking, 1999; Schütte et al., 1999). In this area, we hypothesized that the species may adopt a faster developmental strategy, altering its voltinism in respect to central and northern Europe. Additionally, we applied a standardized sampling protocol, in order to obtain reliable indications on population size and age structure over a continuous time period, as sampling gaps (as in Schütte et al., 1999) or low number of sampled individuals (as in Göcking, 1999) can hamper interpretation of results.

The size of the studied larval population reached its minimum just before the appearance of very small larvae. This was most likely a consequence of emergence of late instar larvae coupled with the scarce amount of newly hatched larvae, that have not already fully replaced the emerging population. The highest larval population sizes were reached around October, just before the beginning of the colder season. Most likely, at this point of the season all larvae had hatched and no further larvae emerged, increasing the number of instars and individuals. The subsequent apparent reduction in the size of the larval population during winter months was probably caused by the combined effect of larval mortality and larvae hiding for hibernation (Robert, 1958). The latter would result in a reduced probability of catching larvae.

Minimum larval population densities were reached approximately 1.5 months earlier (beginning of July) than in the population studied in Germany (mid-August) by Göcking (1999). However, the fact that Göcking (1999) had not followed a standardized sampling protocol hampers comparison of results. Additionally, in our population a winter low population density was reached at the end of February 2015, approximately two months before the winter minimum found by Göcking (1999) (end of April). This advance in phenology is probably due to warmer temperatures and lower latitude of our sampling site with respect to the German populations and it seems likely that the start of larval growth begins earlier in Northern Italy. Also in comparison with the data provided by Schütte et al. (1999) for Germany, our population seems to start development earlier in the season, but the wide gaps in the sampling scheme adopted by Schütte et al. (1999) make it difficult to compare the studies.

We also found that the head-width of larvae of the same instar clearly decreased with the advancing growing season, in F-0 to F-2 larvae and in earlier instars. A reduction in size and mass during the season has already been found in many insect species (Nylin & Gotthard, 1998; Rowe & Ludwig, 1991; Sweeney & Vannote, 1978) and also in adults of *C. splendens* (Gallesi, Mobili, Cigognini, Hardersen, & Sacchi, 2016; Hardersen, 2010), but they were never clearly described for larvae of this species. The cause of seasonal size reduction is usually considered an adaptation to the increased growth rate due to ecological factors and seasonality (Johansson et al., 2001; Nylin & Gotthard, 1998; Rowe & Ludwig, 1991; Stoks et al., 2008): typically, photoperiod, warmer water temperatures and other factors correlate to the advancing season and increase growth rates of larvae determining a reduction of size at the subsequent life-history transition (Johansson et al., 2001; Stoks et al., 2008). Probably, the latter mechanism is the cause for the size reduction of bigger larvae; on the other hand, very small larvae are unlikely to be affected by it and their size reduction may be caused by other ecological factors or by their behaviour (Nylin & Gotthard, 1998; Johansson et al., 2001).

We did not observe distinct instar size peaks in the medium-sized instars during autumn–winter, which may be caused by variation in the number of moults between individuals that adopt different growth rates and occur together. Adding the samples from July–August 2014 to each other would also produce this effect, except for the very first instars which vary less in size. Adjustment of growth rate to reach a specific stage in winter may contribute to this phenomenon.

The main results of this work support that *C. splendens* adopts a mostly univoltine strategy also in Northern Italy. Nevertheless, the analysis of larval population structure showed that the majority of the population grew to F-0 and emerged within the beginning of July. Then, in the next samplings, late instars disappeared from the population until the end of August when few F-0 larvae were sampled. The absence of F-0 larvae in July and August implies that larvae did not emerge in this period, resuming emergence only in late August. This two-group emerging pattern is in accordance with the rearing experiment, in which the majority of larvae emerged near mid-July and some emerged near mid-August. The discontinuous emergence pattern is also reflected by two egg-hatching periods in the stream, which could be inferred by the presence of very early instars: one group of small larvae hatched in the first half of July, from eggs laid in mid-May by the early emergers of the season, a second group hatched in the end of October, from eggs laid by late August emergers. Such a discontinuous pattern of emergence has never been described in larval population studies for this species, but it is somewhat inferable from studies based on exuviae or imagos (Hardersen, 2008; Schütte et al. 1999; Westermann, 2002) and by reports of adults also further north (https://artportalen.se/). However, it would be interesting to investigate other sites in Northern Italy to confirm this pattern.

Two possible explanations for the observed discontinuous hatching and emergence could be partial bivoltinism of the population (Ingram & Jenner, 1976; Norling, 1984) or partial semivoltinism (Göcking, 1999). When partial bivoltinism occurs, a fraction of a year cohort develops fast and emerges within the same year; the other part of the cohort develops more slowly to emerge the following year. On the other hand, in the case of partial semivoltinism a cohort splitting occurs but a fraction of a year cohort develops slowly and emerges two years after hatching.

Partial bivoltinism could be in accordance with the population structure observed in the stream, which seems very similar to the pattern observed in *Enallagma aspersum* (Ingram & Jenner, 1976). Observing Figure 1, it seems that all the overwintering population reached maturity in July and emerged. These individuals laid eggs that hatched and produced a new year cohort. The majority of the year cohort developed as univoltine, growing slowly and emerging only the next year. On the other hand, a small early fraction of the new year-class continued the very rapid growth of the first instars and reached maturity, producing the second small peak of F-0, at the end of August and September. These latter individuals laid eggs and produced the second small peak of newly hatched larvae in October, that will emerge the next year. In the case of partial bivoltinism, two emerging periods are expected: a large group of larvae emerges in the beginning of the season, including both the overwintering univoltines and bivoltines, and a smaller group emerges later, consisting only in the second generation of bivoltines (i.e. Khelifa, 2017).

However, partial bivoltinism is not in complete agreement with the results of the rearing experiment: laboratory bred larvae were all collected in March, so they hatched the previous year, nevertheless they showed two main emergence periods. In the case of partial bivoltinism the whole larval population that overwinters in the stream should emerge in the beginning of the season. Afterwards, only the bivoltine fraction of the newly hatched larval population would grow to F-0, producing the second peak of emergence, near September. So, if partial bivoltinism occurred, we would have obtained only one peak of emergence in the laboratory, because we sampled only overwintering larvae.

On the other hand, partial semivoltinism cannot completely be excluded because it could partially agree with some results from the rearing experiment and partially with the observed larval population structure. In case of semivoltinism, the majority of the cohort should be univoltine and emerge in the first half of the next breeding season, taking advantage of more suitable weather conditions and long time before the approaching of winter. But our data showed that some of the larvae hatched also late in the season. These latter larvae probably split into two groups: those that grow fast enough to emerge as univoltine late emergers the next year, and those that develop more slowly, and postpone emergence passing two winters in the stream, becoming the semivoltine part of the cohort. Thus, the individuals emerging before mid-July consists of the univoltine early emergers and the two-year-old semivoltine emergers. Instead, the observed late emergence group should include part of those individuals that hatched late in the previous season and that had grown fast enough to emerge as univoltine late emergers. Late emergers are individuals that are able to reproduce, because in Northern Italy the breeding season lasts until the end of October (Hardersen, 2008; Gallesi, unpublished data), but their fitness may be reduced due, for example, to adverse weather conditions. On the other hand, semivoltine individuals will reproduce under better weather conditions, but are subjected to the risk of increased mortality and predation, because they have to overwinter. It is likely that the adoption of one of these "strategies" is probably plastic and the two strategies are maintained because their fitness success varies between years. During years with a shorter and harsh autumn, late emergers will face high fitness reduction with respect to semivoltine individuals, but in years with an extended growing season, their fitness will increase in respect to semivoltine individuals. Late emergers have not been found in northern populations (Göcking, 1999; Schütte et al., 1999), probably because their fitness may be reduced too much by the shorter extent of the breeding season in Central Europe, so they gain more fitness in postponing emergence to the next year.

This explanation could agree with the breeding experiment because overwintering larvae, those reared in the experiment, are a mixture of univoltines, early and late emergers, together with semivoltines. Early univoltines and semivoltines will emerge early in the season, creating the first observed peak, and the remaining fraction of univoltines will emerge later in the season. However, according to partial semivoltinism, in winter larvae should have been also a group that split from the univoltine late emergers and constituted the 2015 semivoltine part of the population, remaining in the larval stadium until the next season. But the larvae that we bred in the laboratory emerged all within October. Two factors would have prevented observation of the 2015 semivoltines: we selected larvae for the rearing experiment which probably belonged to instars from F-3 to F-5, excluding the very small larvae. This was done to reduce mortality, but this also excluded the last hatching larvae of 2013, that are the most likely to cohort-split, becoming semivoltine and emerging in 2015; additionally, we did not vary the temperature in our experiment, possibly eliminating an environmental factor that could regulate cohort splitting, late in the season.

Semivoltine individuals, however, are difficult to recognize in the observed structure of the population, maybe due to both the presence of generations belonging to different years, that adopt different growth rates, and the small fraction of semivoltines with respect to univoltines.

Additionally, we do not know if late emergers adopt a strategy or if they are only laggards, but it is interesting to note that between mid-July and mid-August no larvae emerged and no F-0 were collected, both in the rearing experiment and in the field study. This might suggest some hitherto unknown fixed developmental strategy.

The complexity of patterns we observed in our study area should not, however, surprise us since larval development is actually quite variable within the genus *Calopteryx*. For example, partial bivoltinism has been found in *C. exul* from North Africa, which actually showed two emerging periods in the field: the majority of individuals emerged early in the season and a smaller second generation of bivoltines emerged late in the season (Khelifa, 2017). On the other hand, partial semivoltinism has been supposed for *C. haemorrhoidalis* in Spain (Ferreras-Romero, Atienzar, & Corbet, 2000), although it was hypothesized from a single big larva found in February (Outomuro & Ocharan, 2011). Additionally, Ferreras-Romero et al. (2000) did not sample in August and September, so it is impossible to observe the eventual occurrence of late emergers, as we found in our species. However, it seems that *C. haemorrhoidalis* overwinters in fewer and earlier instars than our population of *C. splendens*. Finally, *C virgo* was found to reduce the number of generations per year with latitude, adopting a semivoltine life cycle in Germany (Schütte et al., 1999), but a clear univoltine life cycle in south Spain (Outomuro & Ocharan, 2011).

Since we studied only one site, further studies are needed to investigate if this pattern is generally adopted in populations of *C. splendens* in Northern Italy. Moreover, rearing overwintering larvae belonging to a wider number of instars might shed more light on the voltinism of *C. splendens*. Finally, we suggest that populations are sampled from more southern sites to test if the possible pattern of bivoltinism appears clearer, given the reduction of univoltines in the population.

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