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Phenology and temperature-dependent development of *Ceutorhynchus assimilis*, a potential biological control agent for *Lepidium draba*

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Abstract

Lepidium draba (Brassicaceae) is a major concern for agriculture and biodiversity in the western United States. As current control methods do not provide long-term, sustainable solutions, research has been conducted to find biological control agents. *Ceutorhynchus assimilis* is one of the currently investigated candidates. Known as oligophagous in the literature, a specialist clade of this root-galling weevil exists in southern Europe. This raised the question of its ability to survive in colder climates in the target range. We investigated the phenology of *C. assimilis* in the field in southern France (specialist clade) and Romania (generalist clade) and measured various temperature-dependent parameters in the laboratory. In both ranges, weevils were univoltine. Oviposition in autumn started later in France compared to Romania, while mature larvae exited galls (to pupate in the soil) earlier the following year. On average, 25% and 32% of galls from France and Romania were completely below the soil surface, respectively, and this appeared to depend on soil substrate. Weevils transported from France to Romania were able to develop, but at a much lower rate than Romanian weevils. Mortality of overwintering larvae of both clades increased with decreasing temperature and exposure time. At −5°C, lethal times Lt_{50} and Lt_{95} were 15 and 42 days for the specialist clade and 26 and 72 days for the generalist clade. A higher proportion of third instar larvae compared to first and second instar larvae survived. Pupation time at different temperatures did not differ between weevils from France or Romania. A climate match model (comparing winter temperatures) indicated that the specialist clade of *C. assimilis* from France has the potential to establish in some parts of the target range (e.g. Washington, Oregon, California). However, temperature extremes and winters without snow cover will likely limit its establishment unless rapid adaptive evolution takes place.

Introduction

Heart-podded hoary cress, *Lepidium draba* L. (= *Cardaria draba*), is a perennial weed from the family Brassicaceae. It is native to Eurasia (Ball 1964; Jalas et al. 1996) and was probably introduced into North America in the 19th century (Mulligan and Findlay 1974). Since then, *L. draba* has spread from the west to the east coast and from Canada all the way down to Mexico (Rios and Garcia 1998; Gaskin et al. 2005;

USDA NRCS 2014). It is especially problematic in western North America, where it has been declared noxious in 15 US states and three Canadian provinces (Rice 2014; USDA NRCS 2014). It causes important economic losses by invading many economically important crops, including peas, alfalfa, sugar beet. It can spread into adjacent rangeland grass communities and riparian areas, turning large areas into monocultures and becoming a threat to local plant communities, habitat diversity and forage production (Mulligan and Findlay 1974; McInnis et al. 2003; Francis and Warwick 2008). Furthermore, *L. draba* contains glucosinolates that can be a serious threat to livestock health and thus may significantly affect regions with large cattle industries (McInnis et al. 2003). Additionally, *L. draba* can become an alternate host to various insect pests, such as the cabbage seedpod weevil and *Lygus* spp., facilitating their spread into nearby field crops (Cripps et al. 2006).

To date, no long-term management solution for controlling the invasion of *L. draba* has been found. Broad-spectrum herbicides are widely used, but their application is limited if *L. draba* occurs in fields of cultivated or broad leaf crops, which do not tolerate the treatment. For effective control of *L. draba*, often multiple applications are needed, which are highly costly and may threaten surrounding plant communities and habitats (Francis and Warwick 2008). Mechanical control including mowing, grazing or hand pulling can be effective when combined with chemical treatments, but are limited by the large rhizome system of *L. draba* and phenological variability of the weed (Miller et al. 1994; McInnis et al. 2003).

Classical biological control using herbivores from the native range of *L. draba* may offer an environmentally friendly, sustainable control option. Several herbivores associated with *L. draba* in Europe, including the weevil *Ceutorhynchus assimilis* (Paykull) [= *Ceutorhynchus pleurostigma* (Marsham)] (Coleoptera, Curculionidae), show potential for biological control and are currently investigated at CABI Switzerland on behalf of a consortium of mid-western US states (Idaho, Montana, Oregon, South Dakota and Wyoming), British Columbia and two Federal agencies (BLM and USDA-APHIS) (Hinz et al. 2014). The phenology of *C. assimilis* is still not well understood and was only partially studied in southern France (Fumanal et al. 2004a). Adults lay their eggs in young, soft roots of the plant, causing the formation of galls, in which the weevil larvae feed and mature. Development occurs from autumn to early spring. Mature larvae exit the galls in spring and pupate below the soil surface.

Gall making insects, such as *C. assimilis*, are generally regarded as promising biological control agents, as they often show a high host specificity due to their intimate relationship to their host plant (Harris and Shorthouse 1996). *Ceutorhynchus assimilis* was first considered as a potential biological control agent for *L. draba* by Fumanal et al. (2004a). Previously, the weevil has been assumed to have a broad host range (Hoffmann 1954), but preliminary host-specificity tests showed that a population found in southern France was only able to complete larval development on *L. draba* (Fumanal et al. 2004a). Molecular analyses confirmed the existence of four distinct clades of *C. assimilis* (Fumanal et al. 2004b). Two of these clades have a broad host range, whereas the other two seem to be specialized on *Brassica napus* L. and *L. draba,* respectively (Bon et al. 2008). So far the *L. draba* specialist clade was only found in warmer climates of southern France and Italy, whereas the generalist clade is distributed throughout Eurasia and northern Africa (Dennis 1987). In some areas, for instance the French Rhone valley, specialist and generalist *C. assimilis* coexist, but specialists become noticeably more abundant in southern Europe. Both clades are believed to have separated around 20 000 years ago after the last glacial maximum, when climatic refuges were found in the southern parts of Europe (M.C. Bon, unpublished data).

A pre-requisite for a successful biological control agent is that it establishes and builds up populations high enough to have a significant impact on the target weed (Gassmann 1996). The population growth of arthropods depends on their rate of development, fecundity and length of reproductive life (Bale 2002). These reproductive parameters can be compromised by various factors, of which temperature often is the most limiting one (McClay 1996; Kittelson 2004; Olfert et al. 2004; Olfert and Weiss 2006a,b; Van Lenteren et al. 2006). In fact, high overwintering mortality due to poor pre-adaptation of the agent to the winter conditions in its area of release is assumed to be among the leading causes of failure in classical biological control programmes (Stiling 1993). In the current example, investigating overwintering survival is particularly important as the specialist clade of *C. assimilis* from southern France is considered for releases in the continental climate of western North America.

The objective of this study was to investigate whether the specific clade of *C. assimilis* from southern France is able to successfully overwinter in the continental climates of the potential area of release in North America. First, we studied the insect's phenology in southern France and subsequently investigated

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the temperature tolerance of immature stages and adults in a series of laboratory studies. Second, to estimate the potential of the specialist clade from southern France for adaptation to continental climates, we compared its temperature-related characteristics to the generalist clade from Romania. Third, weevils were relocated from southern France to Romania to investigate their survival and development. Finally, we developed a simple climate match model to compare winter temperatures in France and Romania with North America and to predict areas of likely persistence in North America.

Materials and Methods

Phenology, attack rate and gall location

To investigate the phenology of *C. assimilis*, natural stands of *L. draba* infested with *C. assimilis* were sampled once a month in two different regions in Europe: the area of Montpellier in southern France (February 2013 to February 2014) and the area of Iaşi in north-eastern Romania (October 2013 to March 2014) (Table S1). In southern France, plants were collected monthly at three of four sites, according to the availability of *L. draba* plants; in Romania, a single site was sampled throughout the season. Sampling was conducted in transects, ranging from 20 to 30 m in length and 10 to 20 cm in width, depending on plant density. All plants within transects were collected. Due to high patchiness of *L. draba* plants, transects were occasionally subdivided to be able to collect approximately 100 plants per site at each sampling date. On three occasions, these transects were divided into 2 m long partitions to check whether the attack rate of *C. assimilis* would be dependent of the plant density. All plants were transported (Montpellier) or sent (Romania) to the University of Fribourg in Switzerland, where they were stored at 4°C until dissection under a stereomicroscope. Prior to dissection, the root crown diameter, height and gall volume of the plants were measured. Assuming an ellipsoid shape of the gall, its volume was calculated using the formula $V = 4/3 \times \pi \times a \times b \times c$, where a = height, b = width and c = thickness. Shoot height was measured from the root crown to the tip of the longest leaf. As the location of galls in or above the ground could influence the overwintering survival of larvae, galls were divided into three categories: (i) located above soil surface (galls coloured entirely dark green), (ii) partially below (only partially green) or (iii) below soil surface (yellowish, light green). The number of eggs, larvae, dead individuals and exit holes was recorded for each single shoot. The three larval stages were distinguished by measuring their head capsule diameter (see Fumanal et al. 2004a).

To analyse whether attack rates were dependent on plant density, a linear regression model was fitted, including date, site and plant density for each 2-m segment. Gall volume was analysed with linear regression models, including gall volume, total number of larvae and percentage of L3 per gall.

Temperature-dependent oviposition

To test at which constant temperatures *C. assimilis* females will still lay eggs, egg laying females were placed individually into transparent plastic cylinders (15 cm height, 10.5 cm diameter) covered with a gauze lid and provided with a single shoot of *L. draba* potted in soil into a small cup (7 cm diameter, 8 cm high). On 6 December 2013, 60 cylinders were placed in incubators at six different constant temperatures (20.0, 15.0, 10.0, 7.5, 5.0 and 2.5°C; 10 replicates per temperature). On 13 December, all shoots were inspected for signs of feeding or eggs of *C. assimilis*. Feeding was visually scored from 0 (no feeding) to 3 (heavy feeding).

Temperature tolerance of larvae and adults

To obtain a sufficient number of larvae to study the impact of temperature on larval development, 150–270 galls were collected on three occasions from St. Gély in southern France between November 2013 and January 2014 (Table S1). In addition, a single collection of 250 galls was conducted at Iaşi, Romania, in December 2013. At the time of sampling, all three larval stages were present inside the galls. In the laboratory, galls were first equally distributed according to their size, and then, groups of 5–7 galls were placed into small pots (diameter 8.5 cm; height 6.5 cm) filled with moist soil. Galls were placed just below the soil surface, and a short segment of the roots and leaves was left on the galls to avoid desiccation. Pots containing galls were then placed inside incubators and cooled down at a rate of 1.25–2.5°C per day until reaching the desired temperatures of constant 0, −5 or −10°C (Fields et al. 1998; Mathiasen et al. 2015). Equal subgroups of galls were then progressively taken out of the incubators after 1, 5, 12, 19 or 26 days of exposure, and galls were dissected destructively and alive and dead larvae were counted. Larvae found during dissections were placed on wet filter paper and kept at room temperature for 1 hour. If no movement was observed during this period, larvae

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were considered dead. The experiment was repeated three times with galls collected in France and once only with galls from Romania.

A similar experiment was conducted to test survival of *C. assimilis* adults at different temperature regimes. Individuals used in the experiments were taken from a rearing colony maintained at CABI Switzerland (Delémont, Switzerland), originally collected at sites around Montpellier, southern France. Weevils were placed into transparent plastic cylinders (15 cm height, 10.5 cm diameter) covered with a gauze lid and provided with tissue paper for shelter. To estimate cold hardiness, groups of 20 females and males were exposed to −5 and −10°C in January/February 2014. Heat tolerance of adults was tested in July 2014 by exposing groups of ten females and males to 20, 30, 35 and 40°C. Adult survival was checked 1 day after exposure and then in weekly intervals.

Survival rates were calculated by pooling the average survival rates of larvae in each gall of the three replicates for a given time and temperature treatment, making the total number of larvae within one treatment the experimental unit. Combining the three replicates, the number of galls dissected for each temperature was 222 (0°C), 239 (−5°C) and 216 (−10°C). Within one replicate, each measurement event for a given time and temperature treatment consisted of approximately 150 larvae distributed in 10–14 galls. To correct for the proportion of larvae that died prior to setting up the experiment, we used the natural death rate measured in the field from November 2013 to January 2014. The potential influence of larval density per gall on survival was checked with a nominal bivariate analysis with a likelihood ratio test. The potential difference in survival between larval stages was analysed with analyses of variance (ANOVAS). Lethal times 50 and 95 (Lt_{50} and Lt_{95}), at which 50% or 95% of the population died when exposed to a constant temperature, were calculated with a linear regression, fitted over the survival rates obtained with the three replicates of the experiment. Survival of the three larval instars after 26 days exposure to different temperature regimes was compared with a Wilcoxon test. Survival data were arcsine-transformed for analysis.

Temperature-dependent pupation time

To acquire mature third instar larvae, large galls were collected in southern France and Romania at the same sites used to investigate the phenology of *C. assimilis* (Table S1). Galls were placed on a mesh layer large enough to let third instar larvae pass and covered with moist tissue paper to avoid desiccation. One-litre plastic containers were placed underneath the mesh to collect dropping larvae. Emerging larvae were collected daily and placed individually into vials (5 × 1.2 cm), filled with a mix of moist soil and vermiculate and closed with a plastic lid. Two small holes were poked into each lid to allow air circulation and avoid fungal growth. Vials containing individual larvae of either the French specialist or Romanian generalist clade were then placed inside environmental chambers set at constant temperature of 4, 7, 10, 12, 15 or 20°C. Approximately 50 replicates were completed for each temperature treatment. Vials were checked daily for adult emergence. The development data from each set of temperatures were used to construct a degree-day model, which assumes that there is a linear relationship between development rate (1/days) and temperature above a minimum development threshold: $1/d = a\ (bT)$, where $1/d$ is the rate of development and d the duration of development (days), a is the intercept, and b is the slope of the linear function (Campbell et al. 1974).

Pupation time of both clades was compared using a Student's *t*-test and successful emergence rate with a likelihood ratio test. The developmental threshold for pupation was extrapolated using a linear regression. Day-degrees were calculated using the reciprocal of the slope (Hart et al. 2002).

Pupation depth

As pupation depth could be an important factor for overwintering survival, we investigated at which depth larvae of *C. assimilis* pupate by placing individual third instar larvae (from a laboratory rearing of the specialist clade at University of Fribourg) into vials (5 × 1.2 cm) filled with 4 cm of sifted garden soil, which was slightly compressed to simulate field conditions. As pupation depth might differ due to soil conditions or temperature, soil with either high (adding 1 ml of tap water per vial) or low (no water addition) moisture content was prepared and kept at three different constant temperatures (10, 15, 20°C). Fifteen vials were prepared for each treatment, resulting in 90 vials total. After 10 days, the soil was carefully removed and the depth at which weevil cocoons were found was recorded as classes (0–5 mm, 5–10 mm, 10–15 mm, etc.).

A two-way ANOVA was run to analyse the effect of temperature and humidity on pupation depth of the larvae. All data were analysed using JMP 11 (JMP®, Version Pro 11.1.1).

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Survival and development of the French specialist clade in Romania

To investigate whether weevils of the specialist clade from southern France (Mediterranean climate) would be able to survive and develop in continental climates, 18 females and 9 males of *C. assimilis* were relocated in October 2013 from Montpellier in southern France to Iaşi in north-eastern Romania, where climate conditions are similar to the potential areas of release in North America (e.g. Colorado, Idaho, Montana, Oregon, Wyoming). Weevils were then placed on nine gauze-covered pots (two females and one male per pot) containing 10–16 unattacked *L. draba* rosettes. As control, five pots were established in the same way with *C. assimilis* collected in the area of Iaşi. On 7 April 2014, plants were checked for galls and from 5 May onwards, plants were monitored daily for adult emergence. In addition, 20 males collected at Montpellier in October 2013 and 20 males from a colony maintained at CABI Switzerland (originally collected at Montpellier) were kept outside under a shelter in cylinders and provided with cut leaves of *L. draba*. Cylinders were established on 25 October 2013 and weevils regularly checked for survival until 7 April 2014.

Climate match model

As a component of the bioclimatic modelling software CLIMEX, the *Match Climates Function* is used to select a location (Home Location) and identify locations in another area (Away Location) that have similar climates (Sutherst et al. 2007; Kriticos et al. 2012). In this study, we used CLIMEX version 3.02 (Sutherst et al. 2007). CLIMEX's regional climate-matching (RCM) function was used to generate Climate Match Index (CMI) values in order to compare the winter climates for two locations (Iaşi, Romania and Montpellier, France) with North America. CMI calculations were based on equally weighted (1) values for minimum, maximum and average temperatures for the period of 3 December to 25 February. To identify the Home Location, a geographic rectangle, 1° latitude by 1° longitude was used to delineate a geographic region around each location (n = 36 grid cells). The regions of Iaşi, Romania, and Montpellier, France, were defined by 46.7–47.7°N and 27.1–28.1°E and 43.6–44.6°N and 3.4–4.4°E, respectively. CMI values can range between 0 and 1, with a value of 1 indicating an exact match with the Home Location. As both CMI values ≥0.7 or ≥0.8 have been used to identify areas that are climatically suitable for species persistence, we defined three CMI ranges, that is 0.5–0.699 where *C. assimilis* persistence is potentially possible but highly unlikely, 0.7–0.799, where persistence is likely and 0.8–1.0, where persistence is highly likely (Robertson et al. 2008; Kriticos 2011; Haye et al. 2013). The CliMond 10 climate data set was used as input into the model (Kriticos et al. 2012). The CliMond data set was developed for species bioclimatic modelling, including both correlative and process-based mechanistic models. The 10′ gridded data set included a hybrid historical data set (based on CRU CL2.0 and WorldClim centred on 1975).

Results

Phenology, attack rate and gall location in the field

At the beginning of October, mostly eggs (>70%) and first instar larvae were found in *L. draba* plants in southern France (fig. 1a). From autumn to early spring, eggs and all larval instars were found. The first exit holes were observed in November. In February, mostly third instar larvae and exit holes were present, and by May, all larvae had left the galls (fig. 1a).

In Romania, second and third instar larvae were already observed in October (fig. 1b). However, the proportion of third instar larvae did not increase over winter and only a small proportion of exit holes was found from December to February. Larval development only restarted in March, when temperatures were increasing again. During spring the proportion of exit holes increased, while the proportion of larvae and eggs steadily decreased. Thus, a univoltine life cycle was observed in both study areas.

In southern France, plant attack rates ranged from 31 to 84% depending on the sampling date. Attack rates were highest in fall and decreased progressively during winter, when newly emerging *L. draba* shoots became more abundant. No effect of plant density on attack rate was found (ANOVA: $F_{1,88} = 0.234$, $P = 0.630$).

On average, 25 ± 7% and 32 ± 5.8% of galls collected in France (n = 2083) and Romania (n = 261), respectively, were completely below the soil surface. The remaining galls were all either partly or completely exposed. In France, the sampling site with the least rocky substrate (St-Clément-de-Rivière 1) had an average of 38% of galls below the soil surface over the whole measurement period, while at the most gravely site (St-Clément-de-Rivière 2), only 15% of galls were below ground (Likelihood ratio test, $P < 0.0001$). Gall size increased with an increasing number of *C. assimilis* larvae per gall (r = 0.339;

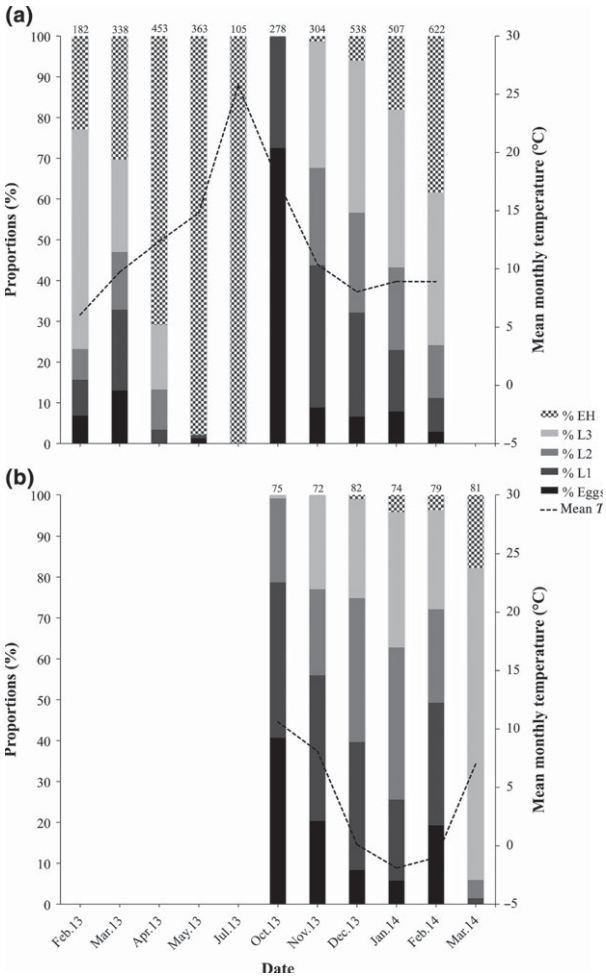

Fig. 1 Phenology of *Ceutorhynchus assimilis* at four field sites around Montpellier, southern France (a), and at one field site close to Iaşi in north-eastern Romania (b) (cf. Table S1 for details). Data are based on dissections of on average 369 (southern France) and 77 (north-eastern Romania) individual plants per month. Data from the four French sites were pooled, as sites were located in close proximity to each other (see Table S1). EH are exit holes, and mean T is the mean monthly air temperature.

$n = 1073$, $P < 0.001$) and an increasing percentage of more mature larvae per gall (r = 0.146; $n = 1258$, $P < 0.001$). Galls contained on average three larvae, but a maximum number of 18 larvae per gall were found.

Temperature-dependent oviposition

Oviposition of French female weevils was highest at 15°C and dropped by more than half at constant 10°C. Four of ten females of *C. assimilis* still laid eggs at 5°C, but the number of eggs per female was much lower than for higher temperatures. Feeding also decreased with temperature and was close to zero below 10°C (table 1).

Table 1 Temperature dependent feeding and oviposition of *Ceutorhynchus assimilis*. Ten females originating from the specialist clade of *C. assimilis* from Montpellier, France, were tested per temperature during 1 week

Temperature (°C)	Average of feeding score (mean ± SE)	Number of females that laid eggs	Number of eggs laid per ovipositing female (mean ± SE)
20	2.7 ± 0.8	10	10.8 ± 2.4
15	2.4 ± 0.8	10	11.7 ± 2.9
10	1.6 ± 1.1	9	4.3 ± 1.0
7.5	0.5 ± 0.8	7	2.8 ± 0.7
5	0.2 ± 0.2	4	1.7 ± 1.2
2.5	0.001 ± 0.003	1	0.2 ± 0.2

Temperature-dependent pupation time

At 20°C, the larvae of the French population took about 21.5 ± 0.1 days from pupation to adult emergence. There was no significant difference between the Romanian and French populations regarding the time required for adult emergence at the different temperatures (*t*-test: $F_{1,247} = 0.006$, $P = 0.938$). The number of days required for adult emergence increased linearly as the temperature decreased. When combining the data for both populations, the linear regression model resulted in the lower threshold temperature $T_0 = 6.8$°C and the thermal constant $K = 286$ ($y = 0.0035x–0.0237$, $R^2 = 0.96551$). Larvae originating from Romania had a slightly higher rate of successful adult emergence (36%) than larvae originating from southern France (28%) ($\chi^2 = 4.783$, $P = 0.029$).

Pupation depth

Cocoons were found in depths from slightly below 0–4 cm, with the majority between 1 and 1.5 cm. Pupation depth was neither affected by temperature (10, 15, 20°C) ($F_{2,73} = 1.846$, $P = 0.162$) nor by humidity (wet/dry) ($F_{1,73} = 2.14$, $P = 0.148$).

Temperature tolerance of larvae and adults

Larvae originating from southern France suffered marginal mortality when exposed to 0°C for 26 days (fig. 2). Fifty per cent of mortality (Lt_{50}) occurred after 110 days of exposure (fig. 2, table 2). At −5°C, Lt_{50} was observed after 15 days of exposure and Lt_{95}

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occurred after 42 days (table 2). At −10°C, the proportion of living larvae dropped rapidly (8% after 5 days), and none of the larvae survived an exposure of 26 days. The calculated Lt_{95} was 18 days (table 2). The apparent increase of survival at −5°C after 5 days may simply be due to natural variation. At 0 and −5°C, a higher proportion of third instar larvae compared to first and second instar larvae survived (ANOVA: $F_{2,1053} = 13.1$, $P < 0.0001$). Larvae originating from Romania showed a significantly higher survival after 26 days of exposure at −5°C (ANOVA: $F_{1,195} = 9.1$, $P = 0.01$) than larvae from southern France (fig. 3). A few Romanian third instar larvae survived 26 days exposure at −10°C, whereas none of the French did (fig. 3). The number of larvae per gall had no significant effect on larval survival (likelihood ratio test, F1,737 = 2.629, $P = 0.105$).

Survival of adults kept at −5°C and −10°C for 22 days at CABI was 74% and 42%, respectively. None of the females survived for more than 30 days at −10°C, while the last males died after 6 weeks. At 20°C, all adults survived 50 days or longer, while at 30°C, survival was high until day 43 (60%), but then rapidly decreased. At 35°C and 40°C, none of the adults survived longer than 8 and 1 days, respectively.

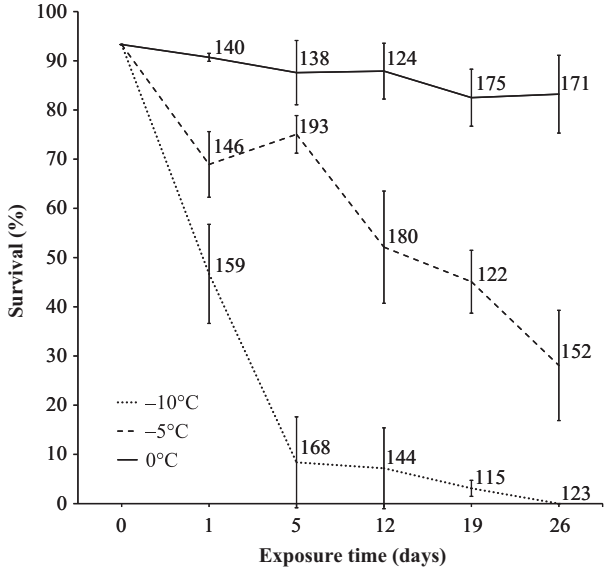

Fig. 2 Survival rates of larvae (L1, L2 and L3) of *Ceutorhynchus assimilis* originating from field sites around Montpellier, southern France, at three constant temperatures during 1, 5, 12, 19 and 26 days. Data are based on an average of 750 larvae per temperature. Error bars are standard errors, based on the three replicates of the experiment.

Table 2 Lethal times (days), Lt_{50} and Lt_{95}, where 50% or 95% of larvae of the French specialist clade of *Ceutorhynchus assimilis* died when exposed to three constant temperatures. Lt_{50} is unknown at −10°C, as the first measure was below 50% survival. Data are based on linear regressions, covering an average of 750 larvae per temperature (cf. text for details). R^2 shows the fit of the regression line

Treat (°C)	Lt_{50} (d)	Lt_{95} (d)	R^2	d.f.	P
−10	na	18	0.47	14	<0.0046
−5	15	42	0.60	14	<0.0007
0	110	234	0.13	14	<0.1832

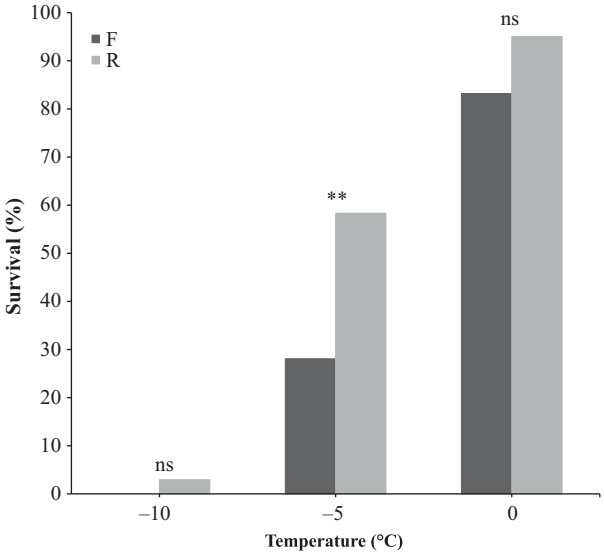

Fig. 3 Survival rates of larvae of *Ceutorhynchus assimilis* originating from field sites around Montpellier, southern France, and from one field site close to Iaşi in north-eastern Romania (cf. Table S1 for details) after 26 days at 0, −5 and −10°C. Only at −5°C the difference is significant (Wilcoxon test, $P = 0.01$). Data are based on an average of 149 (F) and 43 (R) larvae per temperature (cf. text for details). $^{**}P = 0.01$

Survival and development of the French specialist clade in Romania

Plants in three of nine pots infested with weevils from southern France had developed galls, and an average of 5.0 ± 2.7% of plants were galled. In contrast, plants in all five pots infested with Romanian weevils had formed galls and on average 28.0 ± 4.5% of plants were galled. On average, 0.7 ± 0.4 and 4.4 ± 1.2 *C. assimilis* emerged from pots infested with weevils from southern France and Romania, respectively. Of the 20 male weevils originating from France

set up in November 2013 in Romania, 19 individuals survived until 20 December. Survival then continuously decreased and only two individuals were alive by 21 February 2014. Remarkably, the males exposed in the same way from the colony maintained at CABI nearly all survived until the end of January 2014.

Climate match model

Climate-matching techniques have been used to predict potential establishment and distribution of exotic species, including weed biological control agents, in new regions based on their distribution in their native range (Baker et al. 2000; Senaratne et al. 2006). Using CMI values of 0.8 to 1.0 (persistence highly likely), a higher proportion of the mapped area in North America was climatically similar to the winter climates of Montpellier (southern France) than to Iaşi (north-eastern Romania) (21.7% vs. 17.7%, respectively) (Figure S1a,b). This was mostly influenced by the better climatic match of south-eastern and south-central North American winter climates to Montpellier (Figure S1a). In western North America, the model identified climatically similar regions (CMI 0.7–1) to Montpellier winter temperatures along the coast of British Columbia, in Washington, Oregon, south-western and north-western Idaho, wide areas of California and Nevada, northern Arizona and parts of Utah (Figure S1a).

Areas in North America with winter temperatures similar to those in Iaşi (Romania) did not range as far south as for Montpellier, but extended further into the north-western USA, such as Montana, Wyoming and larger parts of Idaho (Figure S1b).

Discussion

Onset of oviposition/post-aestivation activity

In southern France, the specialist clade of *C. assimilis* began laying eggs in late September and continued laying some eggs throughout winter until early spring. Larval development was never interrupted. The continuous oviposition and development in winter was confirmed by laboratory experiments on temperature-dependent oviposition and pupation. Females originating from southern France continued to oviposit, although a reduced rate, at constant 5 and 10°C. The developmental threshold for pupation was calculated at 6.8°C. Average temperatures between November and February in the area of Montpellier, southern France, range from about 5.6–13.8°C (EBCL 2010–2014), which would allow *C. assimilis* to continue oviposition and development throughout winter. In Romania, the generalist clade started oviposition earlier (probably at the beginning of September) and development of eggs and larvae was slow or ceased over winter.

For oviposition and subsequent gall formation, *C. assimilis* depends on the availability of its host plant *L. draba* at the right phenological state, that is newly growing rosettes. It is therefore likely that the stimuli triggering *L. draba* growth and the activity of *C. assimilis* after aestivation are similar. Moisture is most likely the most important factor for the formation of new *L. draba* rosettes after summer (Francis and Warwick 2008). Between June and October, precipitation is generally higher in north-eastern Romania than in southern France (300 mm in Romania vs. 160 mm in France; multiyear averages: Romania, Iaşi weather station 2009–2014; France, USDA, ARS, EBCL 2010–2014). This could trigger earlier regrowth of *L. draba* and therefore earlier oviposition of *C. assimilis* in Romania compared to France and is confirmed by the fact that in southern France, adults ceased aestivation earlier in a watered field experiment compared to the natural field populations (M.C. Bon, unpublished data). The weevil most likely needs another stimulus, for instance, photoperiod, to avoid interruption of aestivation after every rainfall (Tauber and Tauber 1976).

Assuming the onset of oviposition of *C. assimilis* is in fact well synchronized with phenology of its host plant *L. draba,* it is highly likely that the specialist clade of *C. assimilis* from southern France would be able to adapt to different precipitation and temperature regimes during summer and autumn when released in North America, by adapting the start and duration of its oviposition period. A phenomenon which may limit oviposition of *C. assimilis* in the Intermountain West is that in areas or in years where fall precipitation coincides with first frosts, *L. draba* may behave as a hemicryptophyte (Sheley and Stivers 1999).

Cold hardiness/overwintering survival

Temperatures near the freezing point were well tolerated by the French specialist *C. assimilis* clade. At 0°C, larval survival in the laboratory was similar to survival rates encountered in the field. The Romanian generalist clade showed a slight but significantly higher survival at constant −5°C than the French specialist clade, indicating a local adaptation to colder Romanian winters. Considering the large difference in winter climates between southern France and

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north-eastern Romania, the observed difference in larval survival was surprisingly low. In both populations, mature third instar larvae were better suited to freezing temperatures than first and second instar larvae. The actual temperatures that overwintering larvae inside galls will be exposed to in the field depend on many different factors (McDonald et al. 2000). As the soil acts as an insulation layer, the location of the gall certainly plays an important role for overwintering survival. In the present study, an average of 25% of galls in France and 32% in Romania was located below the soil surface, respectively. The remaining galls were partly or completely above ground and thus were more exposed to air temperatures. However, it remains unclear whether larvae inside more exposed galls indeed suffer higher winter mortality. The higher proportion of galls below ground in Romania could indicate a local adaptation of *C. assimilis* to colder winter air temperatures, but it is also possible that this was the result of different soil types. In addition, snow cover has been shown to be associated with higher insect overwintering survival (Bale and Hayward 2010) and may therefore enhance larval survival of *C. assimilis* during short, extreme cold periods, especially for galls that are not located completely below the soil surface.

Climate match model

Climate-matching techniques have been used to predict the potential establishment and distribution of weed biological control agents (e.g. Dhileepan et al. 2013; Zhao et al. 2015). Our climate match model indicates that the specialist clade of *C. assimilis* from southern France is likely to establish and persist in some areas of North America, where *L. draba* is problematic (such as in Washington, Oregon, south-western and north-western Idaho, wide areas of California and Nevada, northern Arizona and parts of Utah), while in other areas with more continental winter climates (such as eastern Colorado, Wyoming and central Montana), establishment and persistence is possible but highly unlikely (Figure S1). In inland British Columbia and the Canadian Prairie provinces, establishment and persistence do not appear to be likely (white areas in Figure S1). As expected, areas in the USA with a winter climate similar to that of Iaşi (Romania) extend further into the north-western USA, especially Montana, Wyoming and larger parts of Idaho. In contrast to our study, (Dhileepan et al. 2013) calculated the lower developmental threshold for all immature stages of the leaf-tying moth *Hypococsmia pyrochroma* Jones (Lepidoptera: Pyralidae). Logistic difficulties working with an internally feeding insect unfortunately prevented us from successfully collecting such data. It could also be argued that the model we used is constrained by the fact that we only included winter temperatures as a limiting factor (Olfert et al. 2004; Olfert and Weiss 2006a,b). However, our survival tests with adult *C. assimilis* indicate that summer temperatures are unlikely to be a limiting factor for *C. assimilis* persistence if released in North America. Precipitation was not included in the climate match model, because the distribution of the weevil's host plant *L. draba* is limited by adequate rainfall (Francis and Warwick 2008), occurring all across North America except the (extreme) south-eastern part of the USA (USDA NRCS, 2014). As *C. assimilis* is dependent on the presence of *L. draba* and its phenology is well adapted to the phenology of its host plant, precipitation should not be a limiting factor *per se*.

The better performance of the generalist clade from north-eastern Romania could be used as an indication that *C. assimilis* has some potential to adapt to colder climates. This is somewhat confirmed by our pilot transfer test, in which Romanian and French weevils were compared for survival and oviposition in the field in Romania. Results showed that the specialist clade from southern France has low, but nevertheless some potential to survive continental winter conditions. French weevils were only transferred to Romania at the end of October. Winter survival of the specialist clade might have been greater if the experiment would have been started earlier and a higher proportion of larvae would have been mature before the onset of winter.

Recent examples from weed biological control agents show that relatively rapid adaptation after release may occur. For instance, it was reported that the flea beetle *Longitarsus jacobaeae* Waterhouse, introduced from Italy to the USA for control of tansy ragwort (*Jacobaea vulgaris)*, underwent rapid evolution in <30 generations and adapted to high-elevation sites (Mt. Hood, OR) (Szucs et al. 2012). Similarly, the stem-mining weevil *Mecinus janthinus* Germar, introduced from the mild climate of the southern German Rhine Valley in the 1990s for control of yellow toadflax (*Linaria vulgaris*), has only recently (after 20–25 years) been found increasing its population density to damaging levels in continental climates of North America (S. Sing, personal communication). For adaptation to a new environment to occur, it is necessary that a certain genetic variation in ecologically important traits is present in the population of the biological control agent selected for introduction

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(McEvoy et al. 2012). Hence, if the genetic variation of the French specialist clade is preserved in the laboratory culture of *C. assimilis* intended for introduction, a relatively quick adaptation to the new climate in the target range should be possible and areas of successful overwintering will likely expand over time. One such adaptation in *C. assimilis* could for instance be oviposition behaviour. As larvae in galls that are located below the soil surface most probably have a higher chance of overwintering successfully, selection should favour females that lay their eggs further down in the root.

Conclusions for biological control

Based on our laboratory studies and field observations, the differences in temperature-dependent larval survival and pupation time of the specialist and generalist clade of *C. assimilis* were less pronounced than anticipated. In addition, the French specialist clade showed some potential to survive in the continental climate of Romania. Conversely, a climate match model indicated that establishment and persistence of the specialist French clade will be highly unlikely in the continental climates of west-central North America. It should be noted that the model used was relatively simple and did not incorporate developmental thresholds for all immature stages of the French specialist clade of *C. assimilis* or the influence of microclimates, for example snow cover. Furthermore, rapid adaptive evolution as observed for other weed biological control agents may allow the specialist clade from southern France to colonize these areas, as well as higher elevations over time.

Overall, our results justify further research on *C. assimilis* as a potential biological control agent for *L. draba*. The impact of the specialist clade of *C. assimilis* on *L. draba* is currently being quantified at the USDA, ARS European Biological Control Laboratory (EBCL) in Montpellier, France, in collaboration with the USDA, ARS Laboratory in Sidney, Montana, USA. Host-specificity tests, including many native North American Brassicaceae, are being conducted by CABI in Switzerland, the results of which look promising so far.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Winter Climate Match Index (CMI) values for Home Location A) Montpellier, southern France and B) Iaşi, north-eastern Romania projected to North America (Away Location).

Table S1. Sites in southern France (F) and north-eastern Romania (R) sampled during 2013 and 2014 to monitor the phenology of *Ceutorhynchus assimilis*.

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