Effects of temperature and food source on reproduction and longevity of aphid hyperparasitoids of the genera *Dendrocerus* and *Asaphes*

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Abstract Hyperparasitoids of aphid parasitoids commonly occur in (sweet pepper) greenhouses, and can pose a threat to effective biological control of aphids. Here, we studied life history characteristics of laboratory colonies of *Dendrocerus* spp. Ratzeburg (Hymenoptera: Megaspilidae) and *Asaphes* spp. Walker (Pteromalidae) that originated from a commercial sweet pepper greenhouse. We aimed to clarify how these two hyperparasitoid taxa can coexist inside greenhouses. Hyperparasitoids of both taxa have a long lifespan that was extended significantly by food sources that are naturally available in a greenhouse environment, including aphid honeydew and sweet pepper flowers. Differences in sensitivity to decreased or increased temperatures did not appear to explain seasonal patterns in abundance of *Dendrocerus* spp. and *Asaphes* spp. in sweet pepper greenhouses. Instead, *Dendrocerus* spp. may have an advantage early in the season because it thrives on aphid honeydew, while *Asaphes* spp. may do better later in the season because of its long lifespan and extensive reproductive period.

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Introduction

Biological control of aphids with parasitoid wasps (Hymenoptera) is widely applied in greenhouse crops. Parasitoids in the family Braconidae (Aphidiidae) are among the most efficient biocontrol agents of aphids because of their short generation time and high reproduction rates so they can keep up with the very fast reproduction of their aphid hosts. However, these aphid parasitoids also have their own natural enemies, including fungi, predators and hyperparasitoids, which can greatly reduce the efficiency of biological control (Prado et al. 2015).

Hyperparasitoids are hymenopteran wasps that use other parasitoids as their host, i.e. they belong to the fourth trophic level. Obligate hyperparasitoids can only develop in or on another parasitoid, while facultative hyperparasitoids may develop as primary or secondary parasitoids (Sullivan and Völkl 1999). Aphid parasitoids are parasitized by a number of obligate hyperparasitoid species from four hymenopteran families. They either attack parasitoid (pre)pupae in mummified aphids (Pteromalidae and Megaspilidae), parasitoid larvae in live aphids (Figitidae), or can use both strategies (Encyrtidae) (Sullivan and Völkl 1999). Multiple hyperparasitoid species are often found in the same habitat, including greenhouses. *Dendrocerus* Ratzeburg (Megaspilidae) and *Asaphes* Walker (Pteromalidae) were found to be dominant in sweet pepper (*Capsicum annuum* L.) greenhouses in British Columbia, but not in open field sweet pepper, suggesting that greenhouse conditions are favourable to these two taxa (Acheampong et al. 2012), or, *vice versa*, that greenhouse conditions are less favourable to other hyperparasitoid taxa.

Within the genus *Dendrocerus*, most European species are known to parasitize aphid mummies, and most species have a broad host range, i.e. they can parasitize combinations of many aphid species and primary parasitoids (Fergusson 1980). The same is true for the genus *Asaphes*, which has two European species that both parasitize a wide range of aphid-parasitoid combinations. Both taxa are thus generalist hyperparasitoids of aphid mummies, in contrast to larval hyperparasitoids (Figitidae) that are often more specialized (Sullivan and Völkl 1999). *Dendrocerus* and *Asaphes* are hyperparasitoids that attack parasitoid (pre)pupae within mummified aphids, and both taxa have an ectoparasitic and idiobiont lifestyle

(Buitenhuis et al. 2017). *Asaphes* species are known to also host-feed on aphid mummies (Buitenhuis et al. 2017; Keller and Sullivan 1976), a phenomenon that appears to be absent in *Dendrocerus* species, except *D. laticeps* Hedicke (Mitsunaga et al. 2014). Host feeding is known to be associated with reproductive strategies in parasitoids (Heimpel and Collier 1996; Jervis and Kidd 1986), and this has also been shown for hyperparasitoids, for example in *Gelis agilis* Fabricius, a hyperparasitoid of *Cotesia* parasitoids (Harvey 2008; Harvey et al. 2012).

Coexistence of *Dendrocerus* and *Asaphes* species in greenhouses has been reported on several occasions (Acheampong et al. 2012; Bloemhard et al. 2014; Jacobson 2011; Nagasaka et al. 2010). In The Netherlands, *D. aphidum* appears to occur earlier in the season than *A. suspensus* in organic sweet pepper greenhouses (Bloemhard et al. 2014). This trend was also observed in Japan, where hyperparasitism by *D. laticeps* on banker plants in greenhouses occurred throughout the season, while *A. suspensus* Nees was only observed later in the season (Nagasaka et al. 2010). In open sweet pepper fields in British Columbia, *Dendrocerus* appears to arrive earlier in the season than *Asaphes*, with *A. suspensus* only recorded in August and September (Acheampong et al. 2012). However, the opposite pattern may also occur. In a British survey, *A. suspensus* was dominant in one sweet pepper greenhouse throughout the season, while at another site it was dominant until a mid-season insecticidal treatment, whereafter other species were observed (Jacobson 2011).

To better understand how *Dendrocerus* and *Asaphes* species can coexist, and to assess the factors that explain their seasonal trends, we set up a series of laboratory experiments. We first assessed the suitability of parasitized aphids and mummies of different ages as hosts for *Dendrocerus* spp. and *Asaphes* spp. We then evaluated their lifetime reproduction pattern, expecting *Dendrocerus* spp. to start reproduction earlier and *Asaphes* spp. to continue reproduction longer and have a higher lifetime fecundity, based on a recent comparison between *D. carpenteri* Curtis and *A. suspensus* (Buitenhuis et al. 2017). Because lifespan may be an important determinant of reproductive output in these taxa, as it is for some other (hyper)-parasitoids (Jervis et al. 2008), we investigated the effect of food sources on longevity. Here, we focused on food sources that are naturally available to

hyperparasitoids in a greenhouse environment, including sweet pepper flowers and aphid honeydew. We also determined the effect of temperature on hyperparasitoid oviposition and longevity, and we assessed cold tolerance of both taxa. Because seasonal trends suggest that *Dendrocerus* is more abundant earlier than *Asaphes*, we hypothesized that *Dendrocerus* spp. are more cold tolerant than *Asaphes* spp.

Our experiments were performed with laboratory colonies of *Dendrocerus* spp. and *Asaphes* spp. that originated from material collected in a sweet pepper greenhouse in The Netherlands. Although originally identified as *D. aphidum* and *A. vulgaris* (Fergusson 1980; Graham 1969), it became apparent that *D. laticeps* and *D. carpenteri* were also present in our *Dendrocerus* colony and *A. suspensus* was present in our *Asaphes* colony after completion of the experiments (tentative identifications by Frank van Veen at the University of Exeter, UK). Because these species were present in our colonies in unknown ratios, we will further refer to them as *Dendrocerus* spp. and *Asaphes* spp. This will not affect our conclusions because any differences between the two genera will be substantially larger than those within.

Methods

Biological system

For this study, we reared hyperparasitoids on pepper plants infested with tobacco aphids parasitized by *Aphidius colemani* Viereck because we wanted to approach the situation in sweet pepper greenhouses as closely as possible. Sweet pepper plants (*Capsicum annuum* var. Maranello) were grown in a greenhouse and infested with *Myzus persicae nicotianae*, Sulzer when they were approximately eight weeks old. They were placed into large mesh cages (Bugdorm 4S4545, 47.5 × 47.5 × 47.5 cm) holding aphid colonies, for 3–5 days. Then, two to three aphid-infested plants were transferred to a large mesh cage with *A. colemani* parasitoids for one day. Parasitized aphids developed into mummies over the course of the next seven days in another large mesh cage. This 'preparation' of pepper plants with *A. colemani* mummies was done six times per week to maintain a constant supply of fresh mummies for hyperparasitoid rearing. Hyperparasitoid colonies of *Dendrocerus* spp. and *Asaphes*

spp. were started with adults emerged from hyperparasitized mummies collected in 2015 and 2016 in a commercial organic sweet pepper greenhouse in Oosterbierum, The Netherlands. Hyperparasitoids were kept in large fine mesh cages (Bugdorm 4F4545) and provided with water and honey, and pepper plants with *A. colemani* mummies were added daily. Plants were cut several times per week and transferred to emergence cages for development of adult hyperparasitoids. All insects were kept and experiments performed in climatised cabinets or chambers at 22 ± 1 °C, 60–70% RH, and 16:8 h L:D photoperiod.

Experiments

Mummy age

To study the suitability of different *A. colemani* host ages for parasitism by *Dendrocerus* spp. and *Asaphes* spp., late parasitized aphids (one day prior to mummification), early mummies (dead, but not yet fully mummified aphids, with a soft exoskeleton) and mummies of different ages were provided to hyperparasitoids. *Aphidius colemani* emerges from mummies five days post-mummification (13 days post-oviposition), so we used mummified aphids that were parasitized 8–13 days before. One starting batch of mummies was obtained by infesting three five-week-old sweet pepper plants with 170 adult aphids each at day zero. The next day, adult aphids were removed and approximately 300 newly produced nymphs per plant remained. Two days later, 80 *A. colemani* parasitoids were added to each plant for 8 h. Seven days after parasitism, late parasitized aphids were collected and tested in the experiment. Early mummies and part of the fully formed mummies that developed over the next 24 h were tested eight days post-parasitism. Remaining mummies were collected from the plants and stored in a Petri dish kept at 22 ± 1 °C for use on the following five days.

Each test day, five mated *Dendrocerus* spp. and *Asaphes* spp. females of 2–4 days old were placed individually in a special Petri dish with a circular mesh membrane in the lid (10 cm diameter, 4 cm high; Insect Breeding dish, SPL Lifesciences Co. Ltd, Korea, further referred to as insect breeding dish) and provided with a small droplet of honey on the mesh. Five hosts were provided per female. Late

parasitized aphids were provided on sweet pepper leaves, which were stuck to the lid for *Asaphes* spp. because we thought this could enhance parasitism rate. Mummies were placed on a filter paper (Whatman, grade 1) on the bottom of the dish for *Dendrocerus* spp., and on a memo paper stuck to the lid for *Asaphes* spp. After 6 h, hyperparasitoid females were removed. Because early mummies develop into mummies rapidly, exposure time was only 2.5 h. For four weeks, we checked emergence of *A. colemani* or hyperparasitoids daily to determine hyperparasitism rate (the proportion of hyperparasitoids of the total number of 'alive' mummies). Mummies that did not emerge were counted as dead mummies because we did not determine whether they contained *A. colemani* or hyperparasitoid offspring (see "Results" section).

Reproductive pattern and lifetime fecundity

Individual newly emerged hyperparasitoid females were placed in insect breeding dishes, together with three males, and a small droplet of honey provided on the lid. Males were removed after one day. The females were presented with 20 *A. colemani* mummies each day, including the first day, until the female died. In this experiment (and the following ones), mummies were collected eight days post-parasitism because this host age was suitable for both hyperparasitoid taxa (see "Results" section). Mummies were offered on the bottom of the insect breeding dish on a filter paper because offering upside down in the first experiment did not improve parasitism substantially (see "Results" section). Each daily set of 20 mummies was transferred to a Petri dish (9 cm diameter, VWR Scientific) for development of offspring. Emergence and sex ratio of hyperparasitoids was recorded after approximately four weeks. A subset of offspring was weighed on a microbalance (Mettler Toledo MT5, 1 µg accuracy) after drying them in a ventilated oven at 70 °C for three days (152 *Dendrocerus* spp. and 187 *Asaphes* spp. individuals). Developmental time of a subset of offspring was also recorded by monitoring emergence daily (110 *Dendrocerus* spp. and 40 *Asaphes* spp. individuals). Seven *Dendrocerus* spp. and ten *Asaphes* spp. females were used in this experiment, but two *Asaphes* spp. females were excluded from the analyses because one unexpectedly died after reproducing for two days and one did not

produce any offspring for 36 days after reproducing on days two and three.

Oviposition at lower temperature

To evaluate whether oviposition occurs at lower temperatures, we compared parasitism rates of *Dendrocerus* spp. and *Asaphes* spp. females at 5 °C, 10 °C, and 15 °C to the control situation at 22 °C. Individual females of 2–4 days old were placed in insect breeding dishes with five newly formed mummies on a filter paper, and provided with honey. Dishes were placed inside climatised cabinets at the three treatment temperatures under conditions that were otherwise the same as those at 22 °C. After 24 h, females were removed and dishes with mummies were kept at 22 °C to determine parasitism rate after four weeks. Sixteen to 20 replicates were done for *Dendrocerus* spp. at each temperature, and nine to 17 replicates for *Asaphes* spp.

Effect of food sources on longevity

Newly emerged hyperparasitoid females were placed individually in insect breeding dishes. The wasps had access to one of the following food sources: (1) water provided on a small ball of cotton; (2) honeydew from *M. persicae* on a piece of sweet pepper leaf with aphids removed (approximately 16 cm², and excluding the main vain of the leaf); (3) an open flower of sweet pepper, containing floral nectar and pollen; (4) five mummies of *A. colemani* on *M. persicae*; (5) a combination of the honeydew, flower and mummy treatments. These five treatments were compared to (6) a positive control consisting of a small droplet of honey, and (7) a negative control without food or water. Treatments (2) to (6) did not receive additional water. Mortality of the insects was checked daily and food sources were replaced three times per week. We performed 16–25 replicates per food source per hyperparasitoid taxa. Dead hyperparasitoids were collected individually in microcentrifuge vials and weighed on a microbalance as above because larger hyperparasitoids may live longer. Weight data were available for a subset of replicates (77 *Asaphes* spp. females and 97 *Dendrocerus* spp. females).

Effect of temperature on longevity

Methods for longevity measurements at different temperatures followed those for the effect of food sources on longevity. At 10 °C, all insects were provided with a filter paper moistened with water, and honey was applied to half of the dishes. Sixteen replicates were done per hyperparasitoid taxa for each treatment at 10 °C. Light settings at 10 °C were 12:12 h L:D to mimic greenhouse conditions after harvesting the final crop, which is commonly done in November in The Netherlands. In a separate experiment, longevity at 30 °C was compared to 22 °C, in the presence of honey. Sixteen to 18 replicates were done at both temperatures for both taxa. Survival was monitored daily in all experiments.

Cold tolerance

Chill coma and chill coma recovery experiments were performed according to methods by Hazell et al. (2008) with two to four-day old females. For the chill coma, the hyperparasitoids were first kept inside the system at 20 °C for 10 min, then the temperature was lowered to 10 °C in 10 min, and to − 2 °C over the next 75 min. Hyperparasitoid activity was monitored by a video set-up, and the temperature where they last walked was recorded. For the chill coma recovery, the temperature was raised from − 2 to 10 °C over a period of 35 min, and then to 20 °C in 1 min, recording the temperature at which the hyperparasitoids started walking again. For each trial, four hyperparasitoids of the same taxa were used at the same time, and eight replicate sets were done for *Dendrocerus* spp., and five for *Asaphes* spp. In the analysis of the data, 31 *Dendrocerus* spp. females (one individual died) and 20 *Asaphes* spp. females were used. For the chill coma, four *Dendrocerus* spp. females that stopped moving at a temperature above 18 °C were excluded from the analysis, because the cessation of movement may potentially be caused by external factors such as physical damage.

Statistical analysis

Mummy age

Replicates on parasitized aphids and on the oldest mummies were excluded from statistical analyses because these host stages were unsuitable for hyperparasitism. Replicates on early mummies were also excluded because they were exposed for 2.5 instead of 6 h, compromising statistical comparison. Within the mummy age range of 8–12 days post-parasitism (M0–M4), we tested the effects of hyperparasitoid taxa and mummy age (as a continuous variable) and their interaction on the proportion of hyperparasitized mummies and proportion of dead mummies with generalized linear models (GLM) with a binomial error structure and using a logit link function. The first model showed minor overdispersion and the second model minor underdispersion, probably due to the low number of replicates per hyperparasitoid taxa per host age (N = 5; models with an estimated dispersion parameter yielded qualitatively similar results).

Reproductive pattern

In the lifetime fecundity experiment, we determined longevity, realised fecundity (i.e. total offspring), median and maximum daily fecundity, first day of oviposition, oviposition period, and post-oviposition period. These data were not normally distributed and the effect of hyperparasitoid taxa was tested with a permutation test to compare medians for each parameter (R package rcompanion, function percentileTest, r = 10000) (Mangiafico 2018). This test was also used to compare median developmental time of offspring between taxa. Effects of taxa and sex and their interaction on dry body mass were tested with a two-way ANOVA followed by post-hoc pairwise comparisons (Tukey's honestly significant difference test, further referred to as Tukey's HSD). Finally, we tested per taxa whether longevity and fecundity were correlated with a Spearman rank correlation test.

Oviposition at low temperatures

We excluded the *Asaphes* spp. data from statistical analysis because a substantial number of *Asaphes* spp. females did not reproduce for unknown reasons. The effect of oviposition temperature on the proportion hyperparasitism by *Dendrocerus* spp. was tested with a generalized linear model (binomial error structure, logit link function), including only replicates that produced at least one offspring.

Longevity

We used Cox proportional hazard models to analyse the effect of taxa and food source on longevity (R package survival) (Therneau 2015). Right-censored data were included when a hyperparasitoid escaped or was accidentally killed (11 out of 258 replicates). We first compared the interaction model (taxa (as strata) × food) with the additive model (taxa (as strata) + food). The effect of food sources on longevity was then analysed in separate models for *Dendrocerus* spp. and *Asaphes* spp., followed by pairwise comparisons among food sources based on log-rank tests (using R package survminer) (Kassambara and Kosinski 2018), adjusting for multiple comparisons with false discovery rate (Benjamini and Hochberg 1995). The effect of weight as a covariate was tested in separate models because it was available for a subset of replicates only. Longevity at 10 °C was also analysed with Cox proportional hazard models (taxa (as strata) × food), including censored data (six out of 66 replicates), and followed by pairwise comparisons with exact two-sample log-rank tests (R package coin) (Hothorn et al. 2006). The effect of temperature (as a factor with three levels: 10, 22 and 30 °C) on longevity was also tested with Cox proportional hazard models (taxa (as strata) × temperature), including censored data (four out of 100 replicates).

Cold tolerance

Wilcoxon rank sum tests were used to evaluate whether *Asaphes* spp. and *Dendrocerus* spp. had different cold tolerance and recovery temperatures. All statistical analyses were performed with R version 3.5.0 (R Core Team 2018).

Results

Reproduction

Suitability of different host ages

Neither *Dendrocerus* spp. nor *Asaphes* spp. were able to reproduce in parasitized aphids (supplementary material Fig. S1). *Dendrocerus* spp. was able to reproduce in early mummies but no *Asaphes* spp.

offspring emerged from this host stage. Fully formed mummies at 8–12 days post-parasitism (M0–M4) were suitable for both hyperparasitoid taxa. In these mummies, *Asaphes* spp. had substantially lower parasitism rates (median 0.25) than *Dendrocerus* spp. (median 1.00). Hyperparasitism rate of *Dendrocerus* spp. dropped in mummies 12 days post-parasitism (M4), while *Asaphes* spp. had a more or less constant hyperparasitism rate over mummy ages (GLM, taxa × age: $\chi_1^2 = 36.696$, P < 0.001). The oldest mummies (13 days post-parasitism, M5), were unsuitable for *Dendrocerus* spp., while one of the five *Asaphes* spp. females produced a single offspring. The proportion dead hosts was stable over mummy ages M0–M4 and was slightly higher in *Asaphes* spp. (median 0.20) than in *Dendrocerus* spp. (median 0) (supplementary material Fig. S1, GLM, age: $\chi_1^2 = 0.072$, P = 0.788; taxa: $\chi_1^2 = 3.603$, P = 0.058).

Reproductive pattern and lifetime fecundity

Reproduction started earlier in *Dendrocerus* spp. than in *Asaphes* spp., with six out of seven *Dendrocerus* females reproducing on the first day against one of eight *Asaphes* females. *Dendrocerus* spp. produced half of their offspring within 4–5 days, while the reproductive period of *Asaphes* spp. was much longer. In total, *Asaphes* spp. produced more than twice as many offspring than *Dendrocerus* spp. (Table 1). Realised fecundity was significantly positively correlated with longevity in *Asaphes* spp. (supplementary material Fig. S2, Spearman rank correlation, rho = 0.95, P = 0.001), but not in *Dendrocerus* spp. (rho = − 0.65, P = 0.115). The proportion male offspring was nearly twice as small in *Dendrocerus* spp. than in *Asaphes* spp. (Table 1, P = 0.053). Despite these clearly different lifetime reproductive patterns (supplementary material Fig. S3), medians of none of the measured parameters were significantly different between hyperparasitoid taxa (Table 1, percentile test, all P > 0.05). In contrast, in the longevity experiment without hosts (see below), *Asaphes* spp. lived significantly longer than *Dendrocerus* spp. (Table 1, percentile test, P = 0.001; see also Fig. 2). With hosts, hyperparasitoids of both taxa lived approximately half as long than without hosts, suggesting a cost of reproduction in terms of longevity. Adult dry body weight of offspring was significantly different

Table 1 Overview of parameters measured for *Dendrocerus* spp. and *Asaphes* spp. in the lifetime fecundity experiment, provided daily with 20 *M. persicae/A. colemani* mummies until death

Life history parameter	*Dendrocerus* spp. (N = 7)	*Asaphes* spp. (N = 8)	P-value[a]
Longevity (days)	15 (12–20)[b]	26.5 (5–95)	0.060
Longevity (days) in absence of hosts[c]	30 (16–60)	56 (9–82)	0.001
Realised fecundity	67 (14–107)	143.5 (21–786)	0.078
Median daily fecundity	3.5 (0–10)	7 (2–11)	0.171
Maximum daily fecundity	10 (6–18)	14 (9–20)	0.224
First day of oviposition	1 (1–2)	2 (1–3)	0.613
Post-oviposition period (days)	2 (0–5)	3 (0–16)	0.738
Oviposition period (days)	12 (9–19)	19.5 (3–77)	0.098
Sex ratio (prop. males)[d]	0.37 (0.25–0.84)	0.71 (0.54–0.90)	0.053
Offspring measurements			
Dry weight male offspring (µg)[e]	27.62 ± 1.32 (N = 71)	30.37 ± 0.76 (N = 141)	2-way ANOVA: Taxa: $F_{1,335} = 4.113$, P = 0.043
Dry weight female offspring (µg)[e]	33.44 ± 1.16 (N = 81)	40.96 ± 1.68 (N = 46)	Sex: $F_{1,335} = 46.156$, P < 0.001 Taxa × sex: $F_{1,335} = 3.98$, P = 0.047
Developmental time (days)[f]	12 (10–15)	19 (18–20)	<0.001

[a] P-values for comparing medians of *Dendrocerus* spp. and *Asaphes* spp. (percentile test)

[b] Median and range (min–max), except for dry weight: mean ± SE

[c] Longevity with honey but without hosts (no reproduction); data derived from longevity experiment on food sources (Fig. 2, N = 21 for both taxa)

[d] Replicates that did not produce any daughters were excluded (one *Dendrocerus* and two *Asaphes* females)

[e] A subset of male and female offspring was weighed

[f] Developmental time was determined for a subset of 110 *Dendrocerus* and 40 *Asaphes* offspring (mixture of males and females)

between taxa and sexes (Table 1, supplementary material Fig. S4, two-way ANOVA, taxa: $F_{1,335} = 4.113$, P = 0.043; sex: $F_{1,335} = 46.156$, P < 0.001; taxa × sex: $F_{1,335} = 3.98$, P = 0.047). *Asaphes* spp. were heavier than *Dendrocerus* spp. This was particularly clear for *Asaphes* spp. females that were much heavier than males (Tukey's HSD, P < 0.001), while the difference between the sexes was less obvious but also significant in *Dendrocerus* spp. (Tukey's HSD, P = 0.003). *Dendrocerus* spp. offspring developed significantly faster than *Asaphes* spp. offspring (Table 1, percentile test, P < 0.001).

Oviposition at low temperature

Both taxa were able to reproduce when kept at 5 °C for 24 h: one of 16 *Asaphes* spp. females and four of 20 *Dendrocerus* spp. females produced offspring. Overall, parasitism success of *Asaphes* spp. was low in this experiment with only 13 out of 56 females

reproducing over the four temperatures at a median hyperparasitism rate of 0.30 (Fig. 2). *Dendrocerus* spp. hyperparasitism success was higher overall (median 1.0) and positively affected by temperature at oviposition (GLM, $\chi_1^2 = 15.846$, P < 0.001). The proportion *Dendrocerus* spp. females that produced any offspring also increased rapidly with temperature (Fig. 2).

Longevity

Longevity on different food sources

Asaphes spp. and *Dendrocerus* spp. longevity was differentially influenced by the seven food sources that we tested (Fig. 2, taxa (as strata) × food: $\chi_6^2 = 44.34$, P < 0.001). The effect of food sources on longevity was further analysed separately for the two taxa.

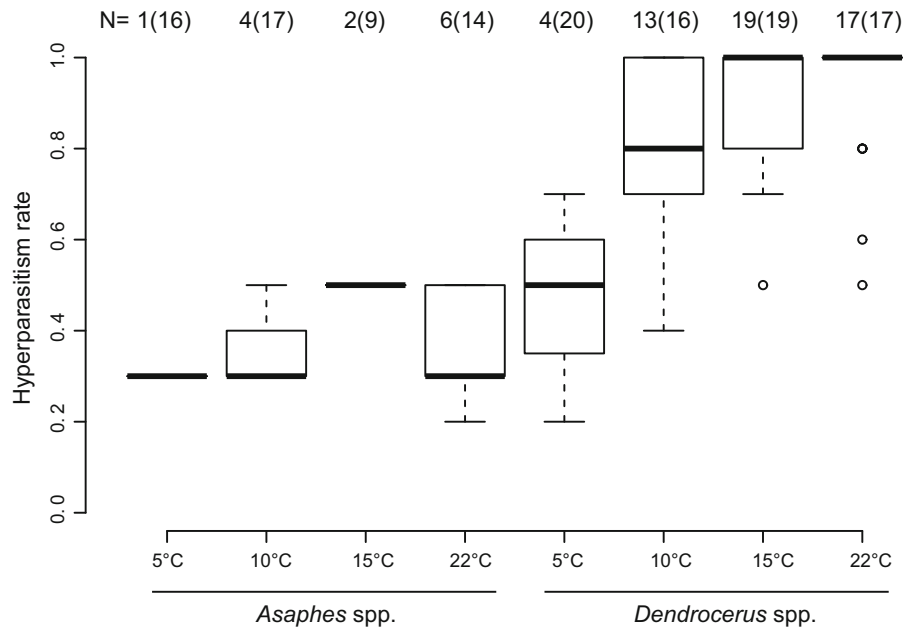Food source had a highly significant effect on longevity of *Dendrocerus* spp. females (Fig. 2a,

Fig. 1 Effect of temperature at oviposition (5, 10, 15 or 22 °C) on parasitism success of *Asaphes* spp. and *Dendrocerus* spp. hyperparasitoids on *A. colemani* mummies developed on *M. persicae*. Numbers above boxplots indicate how many females produced at least one offspring, with the total number of females exposed to hosts in brackets. Females that did not reproduce (i.e. hyperparasitism rate = 0) were excluded. In *Dendrocerus* spp., temperature had a significant effect on hyperparasitism rate

(GLM, $\chi_1^2 = 15.846$, P < 0.001). Results for *Asaphes* spp. were not statistically analysed because of the low number of females that reproduced. In boxplots in Figs. 1, 2, 3, the thick horizontal line marks the median, upper and lower limits of boxes represent the 25th and 75th percentiles of data, whiskers extend to the furthest datapoint from the upper or lower limits to a maximum of 1.5 times the size of the box, and 'outliers' are shown as open circles (default settings for boxplot in R)

$\chi_6^2 = 194.3$, P < 0.001). Longevity was shortest when *Dendrocerus* spp. were given no food or water (control), water or *A. colemani* mummies. Among these three treatments, lifespan on water was significantly longer than that on the control (P = 0.015), while lifespan on mummies was intermediate to both (P ≥ 0.098). *Dendrocerus* spp. females lived significantly longer on sweet pepper flowers or the combination of mummies, flowers and honeydew on sweet pepper leaves (all P < 0.001). Longevity was significantly higher still when *Dendrocerus* spp. was provided with *M. persicae* honeydew on sweet pepper leaves or honey (all P < 0.01).

Food source also had a significant effect on longevity of *Asaphes* spp. females (Fig. 2b, $\chi_6^2 = 136.86$, P < 0.001). Longevity was shortest when *Asaphes* was given no food or water (control) or water only. *Asaphes* longevity increased slightly but significantly on *A. colemani* mummies (P ≤ 0.049). *Asaphes* females lived significantly longer on honeydew of *M. persicae* on sweet pepper leaves, while

longevity on sweet pepper flowers was significantly higher still (pairwise comparison with honeydew, P = 0.048). Longevity on honey was statistically similar to and intermediate between honeydew and flowers (P ≥ 0.088). The combination of mummies, flowers and *M. persicae* honeydew resulted in the significantly highest longevity compared to any other food source (P ≤ 0.028).

Weight data were available for a subset of replicates (97 of 139 *Dendrocerus* spp. and 77 of 119 *Asaphes* spp. females). Analysis of these subsets showed that weight had a marginally significant positive effect on longevity of *Dendrocerus* spp. females ($\chi_1^2 = 3.96$, P = 0.047), while the interaction between weight and food sources was not significant ($\chi_6^2 = 2.52$, P = 0.866). Weight did not have a significant effect on longevity of *Asaphes* spp. ($\chi_1^2 = 0.67$, P = 0.413).
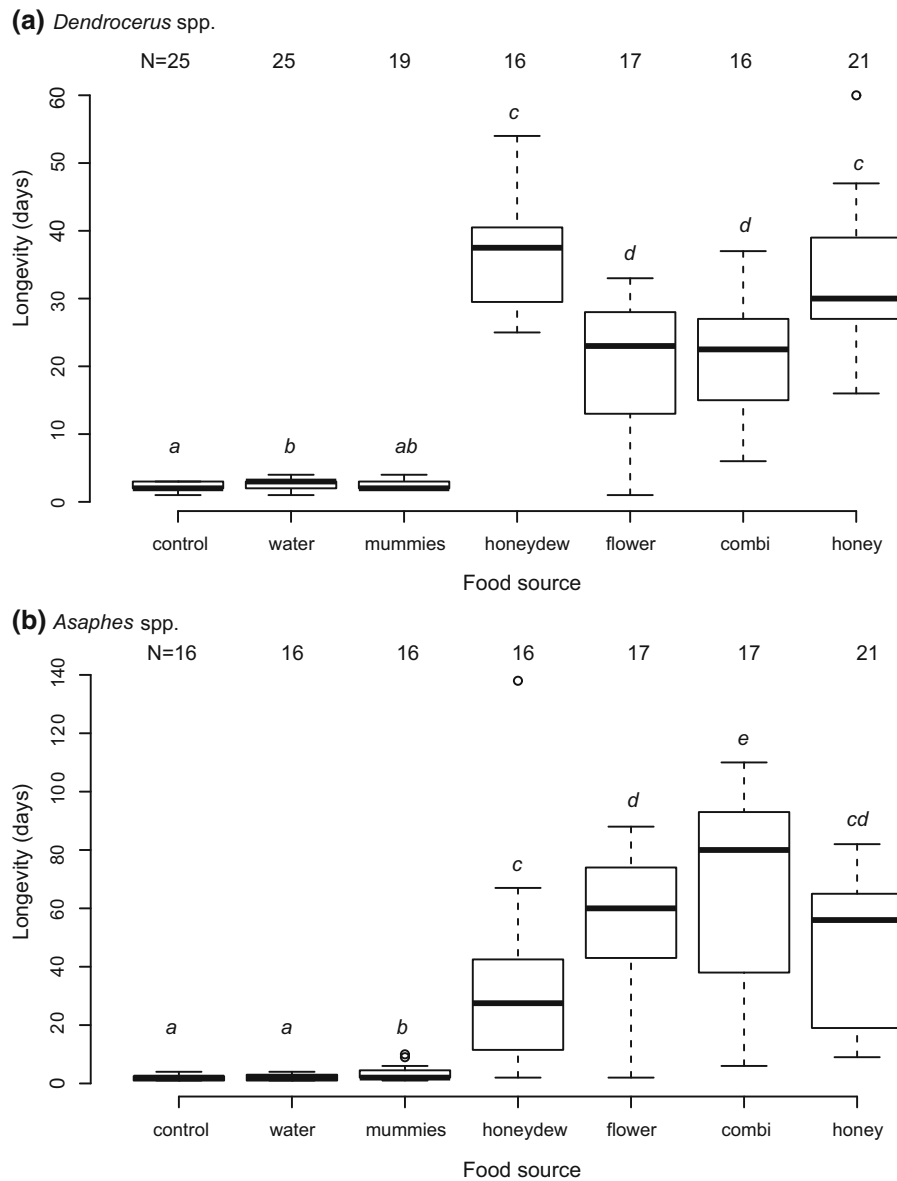
Fig. 2 Longevity of hyperparasitoid females of *Dendrocerus* spp. **a** and *Asaphes* spp. **b** on seven different food sources. Food sources included: control (no food or water), water, mummies (five *A. colemani* mummies developed on *M. persicae*), honeydew (of *M. persicae* aphids on sweet pepper leaves), flower (one sweet pepper flower containing nectar and pollen), combi (combination of mummies, honeydew and flower), honey (a small drop of honey). Numbers above boxes represent the number of replicates. Within species, food source had a significant impact on longevity (*Dendrocerus* spp. $\chi^2_6 = 194.3$, P < 0.001; *Asaphes* spp. $\chi^2_6 = 136.86$, P < 0.001). Different lower-case letters above boxes indicate significant pairwise differences within species (P < 0.05), see supplementary Table S1 for exact pairwise P-values. For an explanation of boxplots, see Fig. 1

Longevity at lower and higher temperatures

At 10 °C, honey significantly increased longevity of both hyperparasitoid taxa (supplementary material Fig. S5, food: $\chi^2_1 = 62.141$, P < 0.001; taxa (as strata) × food: $\chi^2_1 = 1.623$, P = 0.203). On water only, *Asaphes* spp. females lived approximately six days and *Dendrocerus* spp. females 6.5 (exact two-sample log-rank test, Z = − 1.386, P = 0.172). On honey, *Asaphes* spp. females lived significantly longer
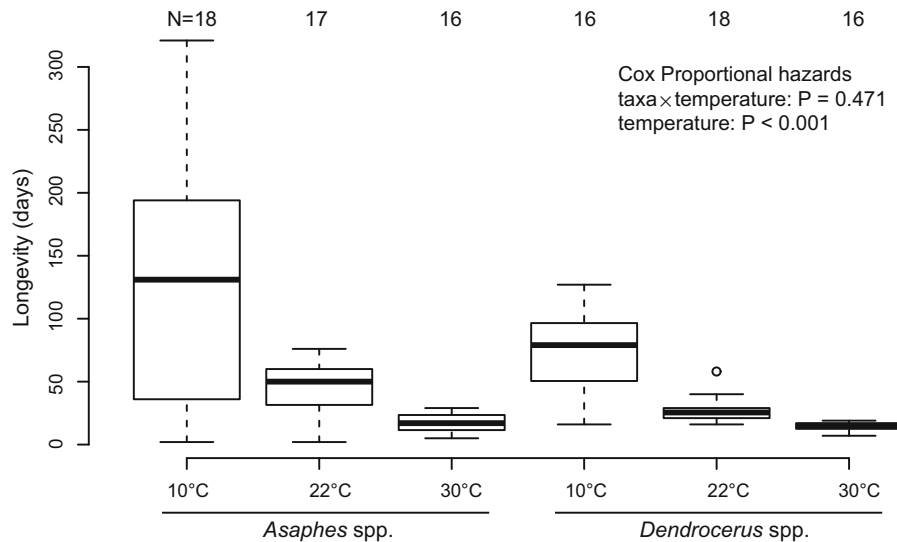
Fig. 3 Longevity of *Asaphes* spp. and *Dendrocerus* spp. females at 10 °C, 22 °C and 30 °C in the presence of honey. Numbers above boxplots indicate the number of replicates. Temperature had a significant effect on life expectancy

($\chi^2_2 = 115.52$, P < 0.001), while there was no significant interaction between taxa and temperature ($\chi^2_2 = 1.504$, P = 0.471). For an explanation of boxplots, see Fig. 1

(median 131 days) than *Dendrocerus* spp. females (median 79 days) (exact two-sample log-rank test, Z = 2.337, P = 0.015).

Survival in the presence of honey at 22 °C and 30 °C were assessed within one experiment, allowing direct comparison (Fig. 3). Longevity at 30 °C was significantly shorter for both taxa ($\chi^2_2 = 115.52$, P < 0.001). Although there was no significant interaction between taxa and temperature (taxa (as strata) × temperature: $\chi^2_2 = 1.504$, P = 0.471), *Asaphes* spp. females appeared to be affected more strongly than *Dendrocerus* spp. by a high temperature of 30 °C because at 22 °C they lived almost three times as long, and at 10 °C more than seven times as long.

Cold tolerance

Asaphes spp. females had a significantly lower mean chill temperature than *Dendrocerus* spp. (Wilcoxon rank test, W = 15, P < 0.001). *Asaphes* spp. only stopped moving at a median temperature below freezing (− 0.17 °C), compared to 7.52 °C for *Dendrocerus* spp. (supplementary material Fig. S6). When raising the temperature, *Asaphes* spp. also resumed moving at a lower temperature (median 5.97 °C) than *Dendrocerus* spp. (median 9.53 °C; W = 52, P < 0.001).

Discussion

Dendrocerus and *Asaphes* species coexist in (sweet pepper) greenhouses as hyperparasitoids of aphid mummies (Acheampong et al. 2012; Bloemhard et al. 2014; Jacobson 2011; Nagasaka et al. 2010). Here, we studied effects of temperature and food source on reproduction and longevity of these hyperparasitoids in an attempt to explain their coexistence and seasonal patterns in occurrence.

Asaphes spp. and *Dendrocerus* spp. females rejected parasitized aphids as hosts as expected because they are known as hyperparasitoids of aphid mummies (Sullivan and Völkl 1999). There was little effect of mummy age on reproductive success of *Asaphes* spp. and *Dendrocerus* spp., except for the oldest mummies (just prior to emergence of *A. colemani*) that were not suitable for development of either taxa. *Dendrocerus* spp. but not *Asaphes* spp. were able to reproduce in early mummies, when the mummy skin was still soft. These findings corroborate previous research on various species of *Dendrocerus*. In *D. laticeps*, mummy age did not affect parasitism success, developmental time, or sex ratio (Mitsunaga et al. 2014). *Dendrocerus carpenteri* can use mummies from right after mummification until just before the adult parasitoid emerges, although emergence of

offspring seemed less successful in the youngest and oldest mummies (Walker and Cameron 1981). Old hosts may also negatively affect developmental time and size of *D. carpenteri* (Otto and Mackauer 1998), parameters that we did not measure here. Correspondingly, *D. carpenteri* preferred mummies of intermediate ages in choice tests (Chow and Mackauer 1999).

The low reproductive success of *Asaphes* spp. in the mummy age experiment may be explained from its lifetime reproduction pattern because we used females that had not been exposed to hosts before. Indeed, while *Dendrocerus* spp. starts reproduction immediately upon emergence, most *Asaphes* spp. females produce their first offspring after a few days (Table 1) (Buitenhuis et al. 2017). This difference may be associated with different patterns of egg maturation in these hyperparasitoid taxa. While *D. aphidum* Rondani emerges with approximately 15 mature eggs (Araj et al. 2008), our own observations suggest that *Asaphes* spp. females emerge with only a few. Host feeding in *Asaphes* may contribute to egg maturation and high fecundity over its lifetime (Buitenhuis et al. 2017), although the effect on egg load has not yet been studied. In other parasitoid species, host feeding can indeed influence longevity and/or reproductive success substantially (Burger et al. 2005; Heimpel and Collier 1996; Jervis and Kidd 1986).

Realised fecundity of *Asaphes* spp. and *Dendrocerus* spp. was in the range recorded in previous studies, confirming that *Asaphes* generally produces more offspring over its lifetime than *Dendrocerus*. Fecundity appears to vary considerably in these hyperparasitoid taxa, both between and within studies. For example, *D. carpenteri* produced 154 offspring at 20 °C on potato aphids parasitized by *A. nigripes* Ashmead (Buitenhuis et al. 2017), but just half as many at 19 °C on pea aphids parasitized by *A. smithi* Sharma & Subba Rao (Walker and Cameron 1981). When *D. laticeps* was kept on honeydew of parasitized aphids, their lifetime fecundity on peach aphids parasitized by *A. colemani* was < 50 at 25 °C (Mitsunaga et al. 2014). *Asaphes*' fecundity appears to be even more variable, ranging from 50 (Mackauer and Volkl 1993) to over 800 for *A. suspensus* (Buitenhuis et al. 2017), and over 1000 for *A. vulgaris* Walker (Brodeur and McNeil 1994). Between studies, variation in fecundity may be due to the exact combination of aphid, primary parasitoid and hyperparasitoid species. Additionally, the food source

offered to hyperparasitoid females likely has a strong impact on their longevity, and may have consequences for reproduction (Jervis et al. 2008), particularly in *Asaphes* because fecundity and longevity are positively correlated. Alternatively, higher temperatures may be less suitable for *Dendrocerus* and result in lower numbers of offspring (Buitenhuis et al. 2017; Mitsunaga et al. 2014). In this study, we therefore evaluated the effects of both factors—food source and temperature—on longevity.

Hyperparasitoid longevity was strongly influenced by food source, with the presence of sugar (in honeydew, nectar or honey) significantly enhancing longevity compared to food sources without sugar (water or mummies). *Dendrocerus* spp. females survived the longest on aphid honeydew, and this was the only food source on which they lived longer than *Asaphes* spp. Interestingly, a recent study showed that *D. aphidum* lives longer on several typical honeydew sugars, than on melibiose, a sugar that only occurs in floral nectar (Goelen et al. 2018). Moreover, *D. aphidum* consumed the highest amounts of melezitose, a sugar that is primarily found in aphid honeydew and rarely in flowers. The congeneric *D. carpenteri* responds behaviourally to aphid honeydew, which served as an arrestant cue (Buitenhuis et al. 2004). Combined, these findings suggest that *Dendrocerus* species are well adapted to honeydew. This adaptation may contribute to their success early in the growing season in greenhouses when sweet pepper plants do not flower yet and honeydew may be the only food source available.

Longevity of *Asaphes* spp., on the other hand, was highest on a combination of mummies, sweet pepper flowers and aphid honeydew, i.e. the 'complete' suite of food sources available in a greenhouse environment. In contrast to *Dendrocerus*, *Asaphes* can feed on aphid mummies, thereby gaining proteins or free amino acids from host haemolymph (Jervis and Kidd 1986). These nutritional elements are required for egg maturation in synovigenic parasitoids (Le Ralec 1995), and may also enhance life expectancy (Heimpel and Collier 1996). Apparently, a combination of host food and sugars derived from non-host food sources increases life expectancy of *Asaphes* spp. most, a phenomenon that has been described in detail for the parasitoid *Aphytis melinus* DeBach (Collier 1995).

Our experiments demonstrate that *Asaphes* spp. and *Dendrocerus* spp. do well at low temperatures.

Females of both taxa oviposited at 5 °C, although only a few females successfully produced offspring. Oviposition success increased rapidly with temperature for *Dendrocerus* spp. Survival at 10 °C in the absence of honey was only approximately one week for both taxa, but humidity was not controlled in our climate chamber and adding water twice per week possibly led to sub-optimal humidity. With honey, longevity of hyperparasitoid females was much higher at 10 °C compared to 22 °C. *Asaphes* spp. in particular lived extremely long at this low temperature, with a third of the females living for more than six months. Coma chill experiments showed that *Asaphes* spp. females stopped moving at just below 0 °C and *Asaphes* spp. was thus more cold tolerant than *Dendrocerus* spp. that stopped moving between 7 and 8 °C. Together, these findings suggest that hyperparasitoids of both taxa are able to survive the period between the final harvest, generally in November in Dutch sweet pepper greenhouses, and planting the new crop, between mid-December and early January. During this time, the greenhouses are not heated and temperatures may drop to 5 °C, close to the ambient temperature in the Netherlands in December. If it can be demonstrated that hyperparasitoids indeed overwinter in empty greenhouses, this could explain the early presence in the year noted by Bloemhard et al. (2014), where *D. aphidum* was already observed in February and *A. suspensus* in March. However, our results do not support the hypothesis that *Dendrocerus* spp. are less affected by low temperature and therefore appears earlier in the season. Alternatively, *Asaphes* spp. may become more dominant later in the season because it copes better with high temperature. Again, our findings do not support this hypothesis. Lifespan of both hyperparasitoid taxa was much reduced at 30 °C compared to 22 °C, and this reduction seemed stronger in *Asaphes* spp. than in *Dendrocerus* spp. Nevertheless, it remains to be studied how these hyperparasitoids respond to more realistic summer greenhouse temperature regimes that are variable and include extremes above 30 °C.

In summary, experiments with two laboratory colonies of *Dendrocerus* and *Asaphes* species show that these hyperparasitoids are long-lived and thrive on food sources that are naturally available in commercial greenhouses. This could contribute to the persistence of hyperparasitoid problems in protected cultivation, where they may indeed affect the efficiency of biological control (Acheampong et al. 2012; Bloemhard et al. 2014; Gomez-Marco et al. 2015; Schooler et al. 2011). The success of *Dendrocerus* early in the season may partially be explained by its good performance on honeydew that is likely the only food source during this period. Additionally, compared to other abundant hyperparasitoid taxa, *Dendrocerus* may be more efficient at finding its host (Buitenhuis et al. 2005), and is known to forage even at night (Völkl and Kranz 1995). We also observed that *Dendrocerus* spp. females are immediately active in host searching once they emerge as adults—which is not the case for *Asaphes* spp.—and we are further investigating their host searching behaviour. Differential effects of decreased or increased temperature do not appear to explain seasonal patterns of *Asaphes* and *Dendrocerus* presence in greenhouses. Instead, *Asaphes* may do better later in the season due to its long lifespan and long reproductive period. Once it has settled in a greenhouse, *Asaphes* can remain present for a long time and can easily overcome periods of low host availability when sugar sources are available. Understanding the dynamics of hyperparasitoid populations in greenhouses is necessary to develop solutions to hyperparasitoid problems in this environment. This requires monitoring studies with good resolution in time and space to validate suggested trends in greenhouses.

Compliance with ethical standard

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