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Defensive symbiosis in the wild: Seasonal dynamics of parasitism risk and symbiont-conferred resistance

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Abstract

Parasite-mediated selection can rapidly drive up resistance levels in host populations, but fixation of resistance traits may be prevented by costs of resistance. Black bean aphids (*Aphis fabae*) benefit from increased resistance to parasitoids when carrying the defensive bacterial endosymbiont *Hamiltonella defensa*. However, due to fitness costs that come with symbiont infection, symbiont-conferred resistance may result in either a net benefit or a net cost to the aphid host, depending on parasitoid presence as well as on the general ecological context. Balancing selection may therefore explain why in natural aphid populations, *H. defensa* is often found at intermediate frequencies. Here we present a 2-year field study where we set out to look for signatures of balancing selection in natural aphid populations. We collected temporally well-resolved data on the prevalence of *H. defensa* in *A. f. fabae* and estimated the risk imposed by parasitoids using sentinel hosts. Despite a marked and consistent early-summer peak in parasitism risk, and significant changes in symbiont prevalence over time, we found just a weak correlation between parasitism risk and *H. defensa* frequency dynamics. *H. defensa* prevalence in the populations under study was, in fact, better explained by the number of heat days that previous aphid generations were exposed to. Our study grants an unprecedentedly well-resolved insight into the dynamics of endosymbiont and parasitoid communities of *A. f. fabae* populations, and it adds to a growing body of empirical evidence suggesting that not only parasitism risk, but rather multifarious selection is shaping *H. defensa* prevalence in the wild.

KEYWORDS
balancing selection, defensive symbiosis, *Hamiltonella defensa*, host–parasite interactions, symbiont-conferred resistance, trade-offs

1 | INTRODUCTION

Parasites can exert strong selection on host populations by favouring resistant over susceptible individuals (e.g. Briese & Mende, 1983; Duncan & Little, 2007). Yet, both the evolution and maintenance of host resistance may be counteracted by life-history costs (Kraaijeveld et al., 2002; Schmid-Hempel, 2003) or trade-offs with other ecologically relevant traits (Cotter et al., 2004; Polin et al., 2014), which will be contingent on environmental conditions (Kraaijeveld & Godfray, 1997; Wolinska & King, 2009). In addition, the selective advantage of resistance may be short-lived when parasites coevolve with their hosts and develop counteradaptations for overcoming

host resistance. We therefore expect temporal and spatial variation in host resistance to parasites, created and maintained by a range of evolutionary and ecological mechanisms (Duffy & Forde, 2009; Schmid-Hempel & Ebert, 2003).

For parasite-mediated selection to act on resistance traits, these need to be heritable, but not necessarily encoded in the host's own genes. Many organisms house heritable microbial endosymbionts, which live in their host's body and have evolved the ability to protect it against parasites (Ewald, 1987; Florez et al., 2015; Jones et al., 2007). These so-called defensive endosymbionts can be regarded as a resistance trait of the host (Jaenike, 2012). The mutualistic character of defensive symbiosis is conditional on a positive balance between the benefits of protection experienced by the host and the costs of housing the symbiont. Whether symbiont-conferred resistance is selected for or against is therefore a matter of ecological context (Bronstein, 1994; Duffy & Forde, 2009; White & Torres, 2009).

Defensive symbiosis is particularly well studied in insects (e.g. Brownlie & Johnson, 2009; Kaltenpoth, 2009); for example in aphids. In this insect group, at least nine repeatedly occurring facultative defensive endosymbionts are known (Guo et al., 2017). In contrast to obligate symbionts such as *Buchnera aphidicola*, which provides aphids with essential nutrients (Douglas, 1998), facultative symbionts are not necessary for aphid survival under benign conditions. However, they can have protective effects against heat (Chen et al., 2000; Montllor et al., 2002), fungal infections (Lukasik et al., 2013) or other adversities (reviewed e.g. in Oliver et al., 2010). The best-studied facultative defensive symbiont in aphids is *Hamiltonella defensa*, a gammaproteobacterium noted for its ability to protect against parasitoid wasps (Asplen et al., 2014; Oliver et al., 2003; Schmid et al., 2012). Parasitoid wasps oviposit eggs into living aphids, where the developing wasp larva eventually causes the aphid's death. The wasp development may be stopped by endosymbiotic *H. defensa* or, more precisely, by toxins produced by so-called APSE bacteriophages present in the symbiont genome (Oliver & Higashi, 2019). As a result, the presence or absence of endosymbiotic *H. defensa* may determine the survival or death of a parasitized aphid.

In many aspects, *H. defensa*-conferred resistance behaves like a classical resistance trait. First of all, the defensive symbiont is costly to its host: in the absence of parasitoids, *H. defensa*-infected aphids tend to lose out when competing with *H. defensa*-free individuals (Dykstra et al., 2014; Hafer-Hahmann & Vorburger, 2020; Oliver et al., 2008). This suggests a trade-off between resistance and parasitoid-independent fitness of aphids, and indeed the presence of *H. defensa* can shorten aphid lifespan and lifetime reproduction (Vorburger et al., 2013; Vorburger & Gouskov, 2011) or reduce defensive behaviour, leading to higher predation risk in natural environments (Polin et al., 2014). Different *H. defensa* strains provide resistance to different parasitoid species or even genotypes (Asplen et al., 2014; Cayetano & Vorburger, 2015), suggesting that *H. defensa*-conferred resistance is determined by specific host–parasite combinations. Also, *H. defensa*-conferred resistance can elicit rapid parasitoid counteradaptation (Dennis et al., 2017; Dion et al., 2011). Finally, vertical transmission of *H. defensa* from one to the next aphid generation may not be perfect (Dykstra et al., 2014; Rock et al., 2018), but likely represents the most reliable way of transmission for the symbiont (Darby & Douglas, 2003; Vorburger, Siegrist, & Rhyner, 2017). This heritability allows for natural selection to act upon the presence and genetic composition of *H. defensa* at the host level (Hafer & Vorburger, 2019; Hafer-Hahmann & Vorburger, 2020).

Strong and directional parasitoid-mediated selection for *H. defensa*-conferred resistance can indeed be observed within weeks in confined experimental aphid populations (Käch et al., 2018). In the field, *H. defensa*-infected and *H. defensa*-free aphids often occur side-by-side; intermediate symbiont frequencies are typical for real aphid populations and are generally attributed to balancing selection (Oliver et al., 2014). The present study was motivated by the fact that—considering the clear costs and benefits of *H. defensa* and the short generation times of aphids and parasitoids—resistance levels in natural aphid populations may be modulated by spatial or temporal variation in selection on an ecological and thus traceable timescale. In temperate latitudes, we expect pronounced seasonal variation in parasitism risk, as a response to strong seasonal dynamics in aphid population sizes. Initiating in early spring from individuals that overwintered as eggs, aphid populations grow rapidly until early summer, when usually a dramatic mid-season population crash is observed, with subsequent recovery towards autumn. Natural enemies certainly contribute to the mid-season population crash (Karley et al., 2004), and indeed the proportion of parasitized aphids increases towards summer, with a certain time lag relative to the aphid population density (e.g. Kavallieratos et al., 2004; Leclair et al., 2021; Yang et al., 2017). These patterns suggest that there is a period in early spring when aphids are selected primarily for fast reproduction, and thereby against *H. defensa*. In contrast, *H. defensa* infection should be selected for in summer, when parasitism risk is high. We would therefore expect the frequency of *H. defensa*-infected aphids to cycle over the year, with a decline early in the season, and an increase during summer.

This common expectation has been addressed in a small number of studies all focusing on pea aphid (*Acyrthosiphon pisum*) populations, and Smith et al. (2015) indeed found large and consistent shifts in defensive symbiont frequencies in US populations of *Ac. pisum* within short time intervals. In one of these populations, parasitoid-induced mortality was positively correlated with an increase in defensive symbiont frequencies. However, in an extensive follow-up study, there were still large symbiont frequency shifts recorded, but rather than by parasitism, *H. defensa* frequency was best explained by temperature (Smith et al., 2021). Contrary to the large shifts observed in both these studies, Leclair et al. (2021) described infection frequencies of various endosymbionts to be surprisingly stable when monitoring French *Ac. pisum* populations over a whole growing season. They did not find parasitoid abundance to correlate with *H. defensa* presence in general, but with the co-infection of *H. defensa* and *Fukatsuia symbiotica*,

another facultative endosymbiont. The somewhat contradictory results of these three field surveys suggest that hitchhiking and non-additive effects of co-infecting symbionts might mask the parasitoid-mediated selection for resistance, or that there might be additional selective forces acting on *H. defensa* prevalence (Carpenter et al., 2021; Smee et al., 2021; Smith et al., 2015). Heat is a candidate factor for such a second selective factor, considering the results of Smith et al. (2021) and the evidence of *H. defensa*-conferred heat protection reported by Russell and Moran (2006). The emerging complexity calls for additional field studies that scrutinize potentially simplistic expectations under natural conditions and help to refine our knowledge on the key factors shaping the heritable aphid microbiome (Oliver et al., 2014).

With this goal we studied natural populations of the black bean aphid *Aphis fabae fabae* (Hemiptera: Aphididae), a widespread and notorious pest feeding on various agricultural crops and weeds (Blackman & Eastop, 2000). Black bean aphids are commonly infected with *H. defensa* (Vorburger et al., 2009; Vorburger & Rouchet, 2016). We carried out a 2-year field study collecting monthly data on the prevalence of facultative endosymbionts and the risk imposed by parasitoids of *A. f. fabae* at three field sites. We asked whether there were significant patterns in the frequency of facultative endosymbionts in natural *A. f. fabae* populations on ecologically relevant timescales, and in particular, whether the frequency of *H. defensa* correlated with preceding parasitism risk or temperature.

We found that despite a marked early-summer peak in parasitism risk in both study years, the patterns in *H. defensa* frequency are not well explained by parasitism risk. Instead, *H. defensa* frequencies are best described by a model where *H. defensa* frequency positively correlates with the number of heat days that previous aphid generations were exposed to. Overall, our study gives a well-resolved picture of the dynamics of endosymbionts and parasitoids in natural *A. f. fabae* populations, and it supports earlier studies in that the effects of *H. defensa* may extend beyond protection from parasitoids.

2 | METHODS

2.1 | Sampling organization

Over two entire growth seasons (2019 and 2020), we regularly estimated the prevalence of up to nine facultative endosymbionts in *A. f. fabae*, and the risk for these aphids of getting parasitized by parasitoid wasps, at three different sites near Zurich, Switzerland. We generally estimated endosymbiont frequencies and parasitism risk at 4-week intervals, and on two extra time points in May and June 2020 to improve temporal resolution during the period for which the 2019 data indicated a high abundance of parasitoids. Endosymbiont infection frequencies were additionally estimated in late October 2019 and 2020, and in April 2021, in order to have data closely spanning two overwintering periods. The exact sampling dates and sample sizes are provided in Table S1. The three sites *Faellanden*, *Gossau* and *Steinmaur* (map in Figure S1) were between 10 and 30 km distant from each other and covered an area of roughly 2 km^2 each. Located in agricultural areas, they were structurally very similar, comprising fields of various crops interspersed with woody hedges growing along small streams. The European spindle tree (*Euonymus europaeus*), the main winter host of *A. f. fabae*, was abundant in these hedges, as well as the guelder rose (*Viburnum opulus*), which can be used as an alternative winter host (Blackman & Eastop, 2000). All sites included fields of sugar beet (*Beta vulgaris*), an important summer host of *A. f. fabae*, and a second important summer host, the goosefoot *Chenopodium album*, was growing in high numbers as a weed across fields of various crops.

2.2 | Estimation of symbiont frequencies in the aphid populations

Aphis fabae fabae is a cyclical parthenogen with a host-alternating life cycle. In late autumn, sexual morphs mate and lay overwintering eggs on the woody winter host plants. Parthenogenetic and live-bearing females hatch from these eggs in spring. After a few asexual generations on the winter hosts, the aphids migrate to their herbaceous summer host plants in late spring, where they continue to reproduce asexually for multiple generations throughout the summer, until returning to their winter hosts in autumn. To estimate symbiont frequencies in the *A. f. fabae* populations, we collected approximately 60 aphids per timepoint and site within 2–3 days from the reference sampling date (Table S1). We sampled *A. f. fabae* from the summer hosts *B. vulgaris* and *C. album* between May and early October and from its winter hosts (*E. europaeus* and to a small extent *V. opulus*) at the other timepoints (Table S1). At each timepoint and site, we sampled aphids from multiple fields and within fields from host plants that were at least 5 m apart from each other, to reduce the likelihood of collecting identical clones. Each aphid was picked up using a fresh pipette tip and placed in a separate Eppendorf tube, which then was stored at −20°C until further processing. We extracted aphid DNA using a salting out protocol as in Sunnucks and Hales (1996). We then set up separate PCRs using specific primers for each endosymbiont and determined the presence or absence of amplified endosymbiont DNA in each sample using a QIAxcel capillary electrophoresis device. Apart from *H. defensa*, we also tested for the presence of the facultative endosymbionts *Regiella insecticola*, *Serratia symbiotica*, *Rickettsia*, *Wolbachia*, *Fukatsuia symbiotica*, *Arsenophonus*, *Rickettsiella* and *Spiroplasma*. As a control for successful DNA extraction, we confirmed the presence of the obligate symbiont *Buchnera aphidicola* (Douglas, 1998) in each sample using the same diagnostic PCR approach. The PCR protocol and primer information are provided in Table S2. *Wolbachia*, *F. symbiotica*, *Arsenophonus*, *Rickettsiella* and *Spiroplasma* occurred at very low frequency or not at all in the samples from 2019 (Table S6); thus, we no longer screened for them in the 2020 samples and did not analyse their frequency dynamics. The frequency of each symbiont was calculated as the number of aphids infected with the symbiont divided by the total number of aphids sampled, for each timepoint and site.

The nominal subspecies of the black bean aphid, *A. f. fabae*, is part of the *A. fabae* complex (Blackman & Eastop, 2000; Heie, 1986) which comprises several subspecies that are morphologically cryptic but may differ genetically and in their facultative symbiont composition (Jörg & Lampel, 1996; Vorburger, Herzog, & Rouchet, 2017). While the summer host plants *B. vulgaris* and *C. album* are used almost exclusively by *A. f. fabae*, the winter hosts *E. europaeus* and *V. opulus* are also used by other subspecies of the *A. fabae* complex (Blackman & Eastop, 2000; Müller, 1982). To distinguish the individuals belonging to *A. f. fabae* among the samples from winter host plants, we genotyped all aphids collected from these at eight microsatellite loci as described in Coeur d'acier et al. (2004). After PCR amplification (see Table S3 for PCR protocol and primer information), the microsatellite fragments were run on an ABI 3730 automated sequencer and analysed with Genemarker 3.0.1. Among the 1713 successfully genotyped samples, 1692 genotypes occurred singly, nine genotypes occurred twice and one genotype occurred three times. All of them were retained for the analysis. Samples of *A. f. fabae* were separated from genetically distinct samples using an expectation–maximization clustering method implemented in the function *snapclust* from the R package *adegenet* 2.1.5 (Beugin et al., 2018), using genotypes from Vorburger, Herzog, and Rouchet (2017) as references (E. Gimmi, J. Wallisch & C. Vorburger, unpublished). With this approach, 942 aphids sampled from the winter host plants were classified as *A. f. fabae* (923 singly occurring genotypes, eight occurring twice and one occurring three times) and used for the further analyses.

2.3 | Estimation of parasitoid frequency

Aphid parasitoids oviposit single eggs into aphids. The hatched wasp larva feeds on the aphid's body, eventually killing it and pupating within the empty aphid exoskeleton. At this stage, parasitized aphids are recognizable as so-called 'mummies'. To estimate the risk of parasitism by various parasitoids in the field, we exposed laboratory-reared, symbiont-free aphids on sentinel plants as baits to the natural parasitoid community. The bait aphids were then returned to the laboratory and kept until those that had been parasitized in the field turned into mummies. This method allowed us to estimate parasitoid abundance and species composition at each site independently of the levels of *H. defensa*-conferred resistance in the local aphid population (low numbers of mummies at a field site could be the result of low parasitoid abundance, or of a high resistance level in the aphid population at this site). To prepare the sentinel plants, we inoculated 3 weeks old broad bean plants (*Vicia faba*, var. Fuego, height circa 20 cm, pot size 10 × 10 × 15 cm) with 15 adults of one single *A. f. fabae* clone from our laboratory collection (A08-28H$^-$). This clone is free of facultative endosymbionts and therefore susceptible to parasitoids. In a climate chamber (22°C, 16 h light), we let the adult aphids reproduce on the plants for 2 days before removing them, leaving behind approx. 120 aphid nymphs on the plant. The day after, we placed 25 aphid-infested sentinel plants at each of our three field sites, digging them into the soil together with their pot so they would fit into the landscape (Figure S2). After an exposure time of 4 days, the plants were brought back to the laboratory, where we immediately counted the number of aphids remaining on the plant (70 ± 51 SD) and removed any visible animals other than our aphids (especially aphid predators and their eggs, ants, aphid parasitoids and slugs). The plant was then covered with a cellophane bag and returned to the climate chamber. From the initially 1350 plants that we set out in the field, 963 were safely returned to the laboratory with bait aphids on them that could be analysed. The other plants could not be recovered from the field because they were destroyed inadvertently by people, consumed by slugs or the plants were recovered but without remaining bait aphids on them. In August 2019, we could analyse particularly few plants from the sites *Gossau* and *Faellanden* (two and six), since the aphids from all other plants had been predated by hoverfly larvae, but on average, we recovered 18 ± 6 SD plants per timepoint and site. Nine days after bringing the plants back to the laboratory, we counted all mummies that had formed in the aphid colonies and collected them in ventilated plastic dishes. Once hatched, we morphologically determined the parasitoids to species level using the keys of Graham (1976) and Stary (1976). Parasitism risk by each parasitoid species was then calculated as the number of hatched parasitoids divided by the number of recovered bait aphids per plant. For statistical analysis, we used the joint risk of parasitism by any parasitoid species. Even though probably not all parasitoid species are equally susceptible to *H. defensa* (Asplen et al., 2014; Cayetano & Vorburger, 2015; Vorburger et al., 2009), we assume this to be the most robust estimate for selection pressure on *H. defensa*-conferred resistance, considering the restricted number of bait plants and the short bait aphid exposure times.

2.4 | *Hamiltonella defensa* strain diversity

Different strains of *H. defensa* may occur in different individuals of the same aphid species and vary in the level and specificity of protection they confer (e. g. Cayetano et al., 2015; Oliver & Higashi, 2019). Selection by parasitoids could thus also act on the relative frequencies of different *H. defensa* strains, rather than just on the overall prevalence of *H. defensa* (Hafer-Hahmann & Vorburger, 2020; Rossbacher & Vorburger, 2020). To investigate the strain diversity of *H. defensa* and potential seasonal variation in haplotype frequencies, we sequence-typed *H. defensa* of 9 or 10 infected aphid samples per site of one timepoint each in spring, summer and autumn of both years. From these 175 samples, we amplified fragments of the bacterial housekeeping gene *murE* and the *P41* gene of the APSE bacteriophage associated with *H. defensa*, using the primers of Degnan and Moran (2008) and a PCR protocol as for symbiont diagnosis (Table S2). Both sequences allow clear distinction of the *H. defensa* haplotypes 1, 2 and 3 described from *A. f. fabae* by Cayetano et al. (2015), and they are also informative for distinguishing *H. defensa* strains from other species of the genus *Aphis* (Henry et al., 2022). We assume that most of the variation in the protective

phenotypes of *H. defensa* occurs between strains belonging to different haplotypes (Cayetano et al., 2015). PCR products were sent for Sanger sequencing to Microsynth AG. We manually checked the sequences with MEGA 11.0.11 (Stecher et al., 2020) and aligned them in R using *seqinr* 4.2.8 (Charif & Lobry, 2007) and *msa* 1.26.0 (Bodenhofer et al., 2015). Using a neighbour joining method and Tamura-Nei distances, we built separate phylogenetic trees for the *murE* and *P41* sequences using *ape* 5.6.2 (Paradis & Schliep, 2019).

2.5 | Data analysis

All analyses were done in R 4.2.3 (R Core Team, 2019) using R studio 2022.02.3 (RStudio Team, 2020) and *ggplot2* 3.3.5 (Wickham, 2016) for plotting. After calculating the individual frequency of every aphid symbiont in the full dataset, we used Fisher's exact tests to assess whether aphids co-infected with different combinations of two symbionts occurred more or less frequently than expected by chance. We tested only for combinations where the expected number of double infected aphids in our dataset was >5, thus for co-infections of *H. defensa* with each *R. insecticola*, *Rickettsia*, *S. symbiotica* and *Wolbachia* and for co-infections of *R. insecticola* and *Rickettsia*.

We expected to see a non-linear relationship between *H. defensa* frequency and time, due to the seasonally variable selection on *H. defensa*-conferred resistance. To generally test for the presence of a seasonal pattern in the frequency of *H. defensa* or *R. insecticola*, *Rickettsia* or *S. symbiotica*, we therefore used generalized additive models (GAMs) using the R package *mgcv* 1.8–40 (Wood, 2017) with default settings. We modelled symbiont frequency separately for each year as a flexible function of time (day of year) while correcting for differences between sites. The function described by the smooth term should then correspond to the trajectory of symbiont frequencies over time, that is the growing season. We used binomial errors and logit links, except for the *Rickettsia* 2020 data where we switched to quasi-binomial errors to account for overdispersion. We then pursued the analysis for *H. defensa* and *Rickettsia*, the two symbionts whose frequencies showed significant patterns in time.

We expected seasonally distinct *H. defensa* frequencies to be caused by seasonally variable strength of selection by parasitism, and possibly as a result of seasonal differences in temperature. To incorporate temperature and in particular heat (Russell & Moran, 2006) into our models, we used data from a weather station close to our field sites (Duebendorf, Zurich, 47°24′ N, 8°37′ E) provided by the Federal Office of Meteorology and Climatology, MeteoSwiss (a single value per timepoint for all three sites). If there was a cause–effect relationship between either parasitism or temperature and *H. defensa* frequency, the maximum of *H. defensa* frequency should follow upon the maximum of positive selection pressure with a certain time lag, depending on the generation time of the involved species but also on how long the selection on *H. defensa* presence remains positive after having reached its maximum. The clearest possible linear correlation between selection strength and *H. defensa* frequency should then be observed under consideration of this time lag. Because the time lag which results in the highest correlation is system specific (e.g. Blanquart et al., 2017; Dybdahl & Lively, 1998; Nee, 1989) and might not be determined a priori, we compared the explanatory power of parasitism and temperature using a lag of either one or two sampling timepoints. This means that we tested for a correlation between *H. defensa* frequency and parasitism risk 4 or 8 weeks earlier ('lag 1' or 'lag 2'), and for a correlation between *H. defensa* frequency and the number of heat days (max. temperature ≥ 30°C) summed up either within the 4 weeks preceding a sampling timepoint ('lag 1') or within the 8 to 4 weeks preceding a sampling timepoint ('lag 2'). We used generalized linear models with binomial errors and logit link, with *H. defensa* frequency as the response variable. We pooled the data of the two sampling years and used year and site as fixed covariates in addition to the effects of parasitism and/or temperature with lag 1 or lag 2. We calculated AICc and Akaike weights to compare the different models and calculated model-averaged coefficients from the three best models using the R package *MuMIn* v1.47.5 (Bartòn, 2023). We also performed deviance analysis for the null model (only site and year as fixed covariates) and the three best models according to AICc using *Anova* from the R package *car* 3.0-12 (Fox & Weisberg, 2019) with default parameters. Using parasitism estimated 8 weeks before *H. defensa* frequency as an explanatory variable had the consequence that the *H. defensa* frequency estimates of the first two timepoints of each year, as well as the estimates of the two in-between sampling timepoints in 2020 (June 4 and July 6) were excluded from this analysis, because we have no parasitism estimates from 8 weeks before these timepoints (as indicated in Table S1). Similarly, the last two estimates of parasitism risk of each year did not enter this analysis, since we have no *H. defensa* frequency estimate 8 weeks after these timepoints.

To get an estimate of how many aphid generations the time lags we tested may include, we used the day-degree method from Campbell et al. (1974) exactly as used in Smith et al. (2021) for pea aphids. This should be a good approximation also for *A. fabae* (Tsitsipis & Mittler, 1976). Accordingly, one aphid generation may have taken between 21 days (in spring and autumn) and 8 days (in mid-summer), and time lags of 4 or 8 weeks may roughly span up to three and six aphid generations (Figure S3).

Finally, we tested whether *H. defensa* frequency changed during the two overwintering periods using Pearson's χ^2 to test for differences in *H. defensa* frequency between the last sampling timepoint in autumn and the first sampling timepoint in spring.

2.6 | Testing for parasitoid DNA in aphid extractions

Visual inspection of the seasonal pattern of *Rickettsia* frequency revealed its close similarity to the pattern of parasitism risk. We confirmed this unexpected result using a generalized linear model with logit link and quasibinomial errors (to account for overdispersion) with site, year and (contemporaneous) parasitism risk as explanatory variables. *Rickettsia* endosymbionts are known to occur in *A. fabae* and other aphid species (Chen et al., 1996; Zytynska & Weisser, 2016; Zytynska

et al., 2016) but also in many other arthropods, including parasitoid wasps (Pilgrim et al., 2021). We therefore suspected that the *Rickettsia* we found in certain aphid extractions might not be aphid symbionts, but rather symbionts of parasitoids that had been present as eggs or early larvae in the aphids we collected. This could explain the high *Rickettsia* prevalence in our dataset just during the period of peak parasitism. To explore this hypothesis, we first tested for *Rickettsia* presence in 48 field-collected parasitoid samples from different species following DNA extraction as for the aphids and using diagnostic PCR as in Table S2. Second, we checked for the presence of parasitoid DNA in 204 aphid extractions from multiple timepoints and sites, of which 40 (20%) contained DNA from *Rickettsia*. To do so, we used PCR as detailed in Table S4 and the primers from Derocles et al. (2012), which target a mitochondrial 16S sequence of Aphidiinae wasps. PCR products that contained parasitoid DNA were sent for Sanger sequencing to Microsynth AG, and the resulting sequences were compared to GenBank (Sayers et al., 2022) using BLAST (Altschul et al., 1990) to determine the parasitoid species from which it originated. To test for a correlation between the detection of *Rickettsia* and parasitoid DNA in aphid extractions, we used Pearson's χ^2 tests.

3 | RESULTS

3.1 | Parasitoid species and dynamics

We found parasitized aphids on 258 of 963 analysed sentinel plants. A total of 5029 parasitoids hatched from the collected mummies, of which 70% belonged to *Lysiphlebus fabarum*. We further found *Aphelinus chaonia* (13%), *Binodoxys angelicae* (8%), *Praon volucre* (4%), *Ephedrus plagiator* (2%) and four other primary parasitoid species, as well as two secondary parasitoid species (all <1%, Table S5). The low number of secondary parasitoids was expected considering the short exposure time of our bait aphids. In 2019, there was a marked peak in parasitism rates on June 20, mainly driven by *L. fabarum*. In 2020, parasitism risk peaked on June 18 and July 2, again driven by *L. fabarum* but less exclusively so (Figure 1, Figure S4). *A. chaonia*, the second most frequent primary parasitoid, reached its peak frequency 4 weeks after *L. fabarum* in both years. The overall parasitism risk was zero or near zero at the first two sampling timepoints in both years (late March and April) and remained low (2019) to intermediate (2020) after the parasitism peak, that is from mid to late summer (Figures 1 and 2, Figure S4).

3.2 | Facultative symbionts of *Aphis fabae fabae*

In total, 3449 samples of *A. f. fabae* were successfully analysed and contained amplifiable bacterial DNA, as indicated by the detection of the obligate aphid symbiont *B. aphidicola*. *H. defensa* was the most frequent facultative symbiont, with 35% of all aphid individuals infected. *R. insecticola* was the second most frequent symbiont and was detected in 8% of the samples. *Rickettsia*, *Wolbachia* and *Serratia* were detected in 3%, 2% and 1% of the analysed samples. In the 1465 samples from 2019 we did not detect any infections with either *Arsenophonus*, *F. symbiotica*, *Rickettsiella* or *Spiroplasma* (Table S6). Co-infections between *H. defensa* and *R. insecticola* occurred less frequently than expected by chance (expected: 91, observed: 18, $p < .001$ in Fisher's exact test). The numbers of co-infections between *H. defensa* and *Rickettsia*, *Wolbachia* or *S. symbiotica* or between *R. insecticola* and *Rickettsia* did not significantly deviate from the expectations based on the individual symbiont frequencies (Table S7). *R. insecticola* and *S. symbiotica* frequencies did not differ significantly between sites and remained stable over time in both years (Table 1, Figure S5). There were significant patterns in the frequencies of *H. defensa* and *Rickettsia* (Table 1, Figures S5 and S6), which are described in the following paragraphs.

3.3 | Exploring *Rickettsia* frequency dynamics

Rickettsia frequency showed a significant pattern in time, peaking in mid-June and July of both years, but did not differ between sites (Table 1, Figures S5 and S6). *Rickettsia* frequencies are strongly correlated to (contemporaneous) parasitism risk ($\chi^2 = 39.18$, df = 1, $p < .001$), which reflects the coincidence of the peaks of *Rickettsia*

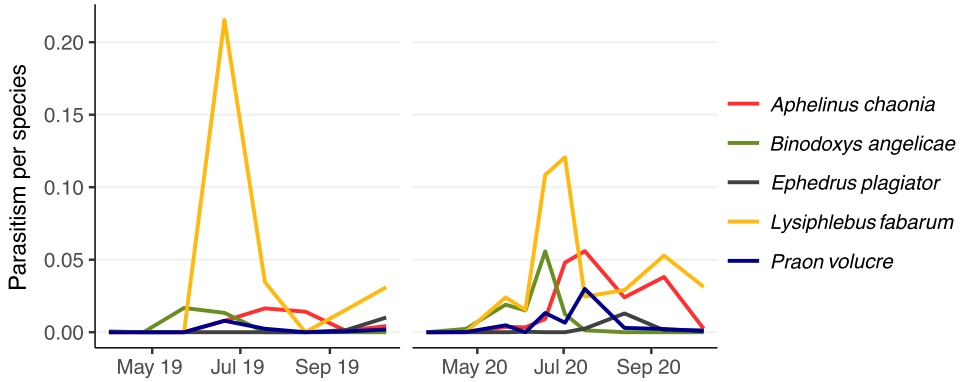

FIGURE 1 Proportion of exposed bait aphids that got parasitized (parasitism risk) as functions of date, separated by parasitoid species and averaged over the three sampling sites.

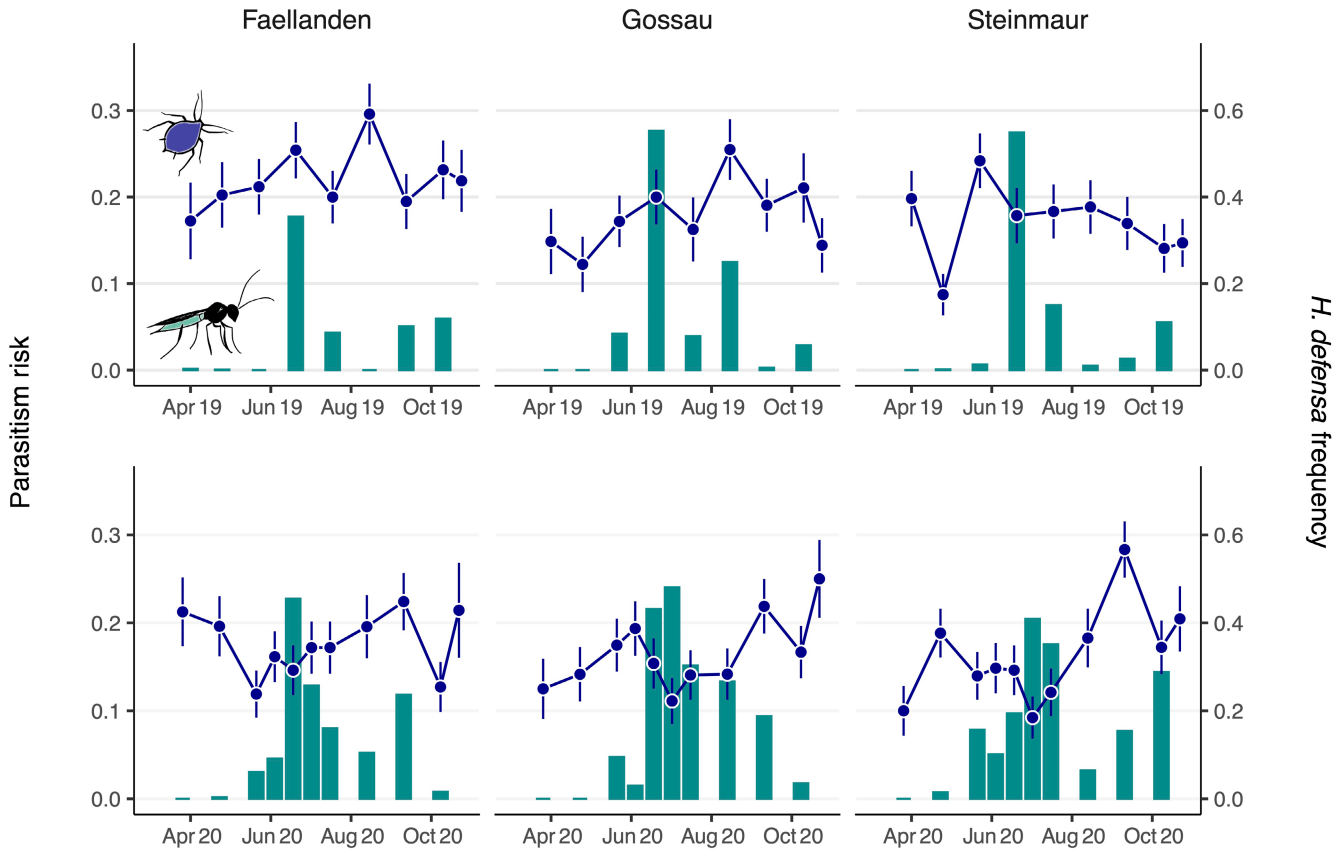

FIGURE 2 *Hamiltonella defensa* frequency and parasitoid frequency at the three sampling sites *Faellanden*, *Gossau* and *Steinmaur* as functions of date. Right *y*-axis, dark blue points: *H. defensa* frequency, that is the proportion of aphids infected with *H. defensa*, with standard errors. Left *y*-axis, bars: parasitism risk, that is the proportion of exposed bait aphids that got parasitized.

TABLE 1 Results from Wald tests for the significance of site (parametric) and time (day of year, smooth term) in generalized additive models for symbiont frequencies, per year.

				Site			s (day of year)			
Symbiont	**Year**	**Family**	**Scale Est.**	**df**	χ^2	***p***	**Edf**	**Ref. df**	χ^2	**Appr. *p***
Hamiltonella defensa	2019	Binomial	1	2	11.54	.003	2.19	2.72	6.00	.112
	2020	Binomial	1	2	0.98	.614	7.30	7.85	31.12	<.001
Regiella insecticola	2019	Binomial	1	2	3.52	.172	1	1	1.31	.251
	2020	Binomial	1	2	2.68	.261	3.84	4.73	5.50	.387
Rickettsia sp.	2019	Binomial	1	2	2.65	.266	5.32	6.09	39.35	<.001
	2020	Quasibin.	2.1	2	F = 3.09	.062	2.64	3.40	F = 3.29	.032
Serratia symbiotica	2019	Binomial	1	2	2.89	.236	1.29	1.52	4.58	.088
	2020	Binomial	1	2	1.58	.453	6.41	7.01	12.79	.072

Note: We used binomial errors and logit links except in the model for *Rickettsia* frequency in 2020, where we used quasibinomial errors to account for overdispersion; *F* statistics rather than chi-square statistics are given in this case and the scale estimator is 2.12. The *p*-value of the smooth term is approximate. Significance of the smooth term (day of year) indicates a consistent pattern in symbiont frequency over time.

frequency and parasitism frequency in June or July of both years (Figure 1, Figure S5). This result is likely caused by aphids collected during the period of peak parasitism (invisibly) carrying parasitoid eggs or larvae, which themselves might be hosts to endosymbionts such as *Rickettsia* (Pilgrim et al., 2021), as our follow-up analysis showed. Firstly, we found *Rickettsia* in 13 of the 48 analysed parasitoid extractions. None of the analysed *L. fabarum* (0/32) but all analysed *B. angelicae* (10/10) carried *Rickettsia*. We also found *Rickettsia* in 1/2 *L. gracilis*, 2/3 *P. volucre* and 0/1 *B. acalephae*. While sample numbers are too low to infer frequency estimates for *Rickettsia* in

these species, the results confirm that *Rickettsia* commonly infects parasitoid species present in the field. Secondly, we detected parasitoid DNA in 37/204 (18%) of the analysed aphid extractions. Among the 35 samples for which the 16S fragment was successfully sequenced, one sequence corresponded to *Aphidius* sp, 26 to *B. angelicae*, six to *L. fabarum* and two to *P. volucre*. *Rickettsia* and parasitoid DNA co-occurred in the same aphid extractions more frequently than expected by chance ($\chi^2 = 77.57$, df = 1, $p < .001$). This supports our hypothesis that some of the *Rickettsia* we detected in aphid extractions were in fact symbionts of parasitoids, rather than aphid symbionts. As not all *Rickettsia*-positive aphid extractions contained parasitoid DNA, it appears that some of the detected *Rickettsia* were still true symbionts of *A. f. fabae*. Nevertheless, the marked peak in *Rickettsia* prevalence that we see in June and July of both years is likely driven by the detection of this endosymbiont in parasitoid eggs or larvae present in part of the sampled aphids.

3.4 | Exploring *Hamiltonella defensa* frequency patterns

Hamiltonella defensa frequency differed between sites but showed no consistent pattern over time in the first sampling year (Table 1, Figure S5). The lack of consistency is due to site *Steinmaur* showing a very different trajectory compared to *Faellanden* and *Gossau* (Figure 2, Figure S5). In *Steinmaur*, we recorded a steep drop and unexpected minimum at the end of April and hardly any frequency changes for the rest of the year, while in *Faellanden* and *Gossau*, *H. defensa* frequencies were lowest in early spring, highest in August and low again in October (Figure 2, Figure S5). In the second sampling year, *H. defensa* frequency did not differ between sites and showed a more consistent pattern in time, in particular an increase at all sites between July and September followed by a drop in October (Table 1, Figure 2, Figures S5 and S6).

Models explaining *H. defensa* prevalence from temperature and/or parasitism with time lag 1 (parasitism 4 weeks prior, heat days within preceding 4 weeks) are clearly inferior to any of the lag 2-models (ΔAICc > 12; Table S8) and were therefore not pursued further. *H. defensa* frequency is best described by a model using the number of heat days in the preceding 8–4 weeks (time lag 2) as only explanatory variable apart from site and year (Table 2: model 2b; Akaike weight = 0.74), followed by a model including heat days with time lag 2 as well as parasitism with time lag 2 (8 weeks prior) (Table 2: model 2c; weight = 0.22). The significant positive effect of heat days with lag 2 is driven by the overall peaks in *H. defensa* frequency observed in August 2019 and September 2020, that is at the end of the summer period in both years (Table 3, Figures 3 and 4). Heat days were almost exclusively recorded in June, July and August of both years (Figure 3). Unsurprisingly, we see a strong positive correlation between temperature and parasitism risk, which also peaked in June and July (Figure S7), making it impossible to fully disentangle their effects. Indeed, parasitism with time lag 2 is a significant predictor of *H. defensa* frequency when temperature is ignored (Table 2: model 2a; Figure 4), but this model receives considerably less support from the data (weight = 0.03), and parasitism is no longer significant when the temperature term is added to the model (Table 2: model 2c). That the number of heat days is a better predictor of future *H. defensa* frequency than parasitism risk is also supported by the fact that the sum of Akaike weights of the two models containing the temperature term is considerably higher than the sum of weights for the two models containing the parasitism term (0.96 vs. 0.25). Accordingly, the model-averaged parameter estimate is significant for the effect of heat but not for the effect of parasitism with time lag 2 (Table 3).

Hamiltonella defensa frequency slightly decreased during both winters and at all sites, but none of the differences between the last sampling in autumn and the first in spring were significant after Bonferroni correction (Table S9).

TABLE 2 Model AICc, Akaike weight and results from analyses of deviance (using type II sums of squares) of the null model (only site and year as fixed explanatory variables), and the three models testing for correlations between *Hamiltonella defensa* frequency and parasitism and/or the number of heat days at time lag 2.

			Deviance analysis			
Model	AICc	Weight	Effect	χ^2	df	*p*
0	221.33	0.001	Site	1.94	2	.380
			Year	11.97	1	.001
2a	214.20	0.033	Site	3.33	2	.189
			Year	13.83	1	<.001
			Parasitism lag 2	9.83	1	.002
2b	207.97	0.743	Site	2.09	2	.352
			Year	10.21	1	.001
			Heatdays lag 2	16.07	1	<.001
2c	210.40	0.220	Site	2.38	2	.305
			Year	10.65	1	.001
			Heatdays lag 2	6.70	1	.010
			Parasitism lag 2	0.47	1	.495

Note: Generalized linear models with logit link and binomial errors were used. Lag 2 means that we test for a correlation between *H. defensa* and the number of heat days within the 8 to 4 preceding weeks, or the parasitism risk 8 weeks before, respectively.

3.5 | Low *Hamiltonella defensa* strain diversity

Of 175 *H. defensa* samples from all three sites and 6 sampling timepoints, 171 samples shared the same sequences for both loci we investigated. These sequences corresponded to the reference sequences of *H. defensa* haplotype 2 (Cayetano et al., 2015). Two samples clustered with *H. defensa* haplotype 1 considering both the bacterial housekeeping gene *murE* and the phage gene *P41*, and two samples clustered with haplotype 2 for their *murE* sequences but contained *P41* sequences that we would have expected for haplotype 3 (Figures S8 and S9). While it is possible that the *H. defensa* samples we analysed show some variation outside the *murE* and *P41* sequences, our results suggest that the *H. defensa* strain referred to as haplotype 2, which was

the prevalent haplotype also in the dataset of Henry et al. (2022), is by far the dominant strain in our sample collection. Hence, the overall *H. defensa* frequency in both years and at all three sites may be virtually tantamount to the frequency of the *H. defensa* haplotype 2. For this reason, we did not analyse *H. defensa* strain diversity any further.

4 | DISCUSSION

Temporal and spatial variation in the balance between costs and benefits may influence resistance levels in natural aphid populations. With our field survey, we explored the hypothesis that seasonally variable parasitism risk provokes fluctuating frequencies of the costly but resistance-conferring symbiont *H. defensa* in populations of *A. f. fabae*. We observed significant changes in the prevalence of *H. defensa* over time, but the temporal dynamics are not well explained by parasitism risk alone, and they just partly correspond to our predictions. On the one hand, there was no decrease in *H. defensa* frequency despite zero or near-zero parasitism risk in the spring period; the expected net costs of *H. defensa* during the period of rapid aphid population growth are thus not evident. On the other hand, we recorded changes in *H. defensa* frequency that were apparently unrelated to parasitoid presence or absence, for example a sudden, concerted drop at the end of the second sampling season. We find that rather than by parasitism risk, *H. defensa* frequency is best explained by the number of heat days that previous aphid generations were exposed to (Table 2).

Discrepancies between expectations based on laboratory experiments and field observations have also been reported from the observational studies on *H. defensa* frequencies in *Ac. pisum*: either a link between parasitism risk and *H. defensa* frequency was not observed at all (Smith et al., 2021), or in some but not all studied populations (Smith et al., 2015), or only when considering *H. defensa* in co-infection with another symbiont (Leclair et al., 2021). In the field cages set up by Smith et al. (2021), high parasitism risk slowed down but could not prevent a decrease in *H. defensa* frequency. In other field experiments again, the protective effect of *H. defensa* against natural parasitoid communities was not significant at all (Narayan et al., 2022), or it manifested in a significantly reduced proportion of parasitized aphids, but without resulting in increased population growth of *H. defensa*-infected compared to uninfected aphids (Hrcek et al., 2016; Rothacher et al., 2016).

TABLE 3 Model-averaged coefficients (full average) of the models 2a, 2b and 2c using parasitism and/or the number of heat days at time lag 2 as explanatory variables for *Hamiltonella defensa* frequency.

Effect	Estimate	Std. error	Adj. SE	z	p
Intercept	−0.681	0.060	0.062	10.99	<.001
Site 1	0.093	0.064	0.067	1.39	.164
Site 2	−0.033	0.065	0.068	0.48	.629
Year 1	0.147	0.046	0.048	3.08	.002
Heatdays lag 2	0.042	0.014	0.015	2.83	.005
Parasitism lag 2	0.169	0.509	0.520	0.32	.745

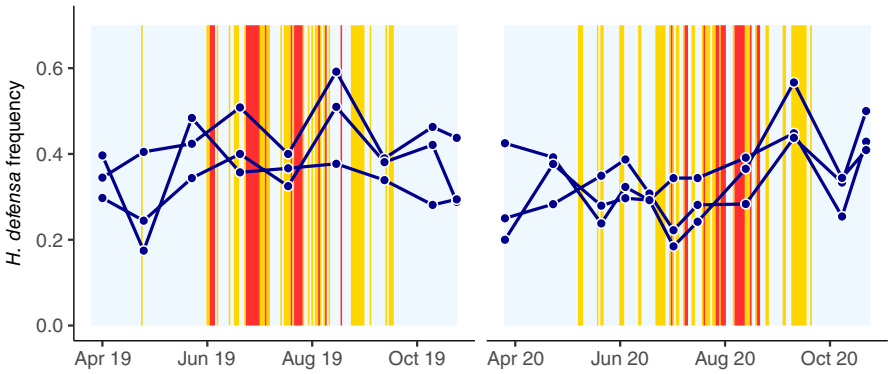

FIGURE 3 *H. defensa* frequency (dark blue points and lines) and temperature as functions of date. Yellow bars indicate days where the maximal temperature was ≥25°C (summer days), red bars indicate days where the maximal temperature was ≥30°C (heat days). *H. defensa* frequency is significantly correlated to the number of heat days within the 8–4 weeks preceding a sampling timepoint.

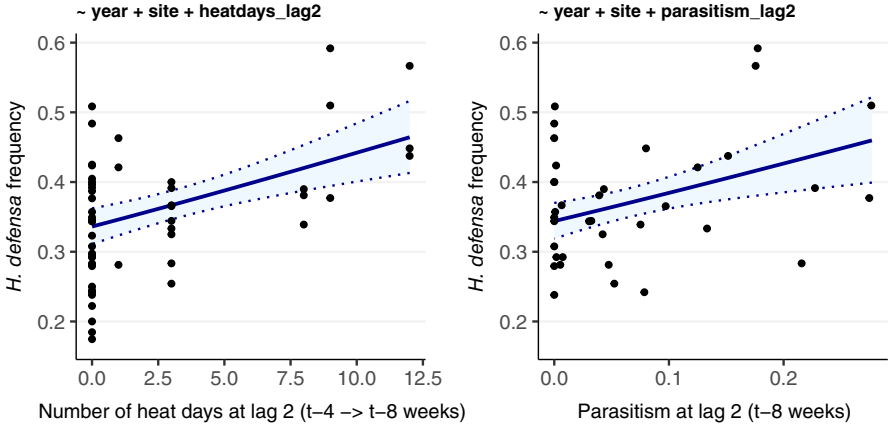

FIGURE 4 Partial effect plots for the model including only the number of heat days at lag 2 (left plot, corresponding values in Table 2b) and the model including only parasitism risk at lag 2 (right plot, corresponding values in Table 2a) to explain *Hamiltonella defensa* frequency. The dotted lines delimit 95% confidence intervals of the regression line.

Even though not unprecedented, the weakness or lack of a relationship between parasitism and *H. defensa*-conferred resistance in the field is surprising. Parasitoid-mediated selection has been documented numerous times from laboratory experiments (e.g. Herzog et al., 2007; Käch et al., 2018; Oliver et al., 2008; Xie et al., 2015), but also from a large-scale field experiment under semi-natural conditions (Ives et al., 2020). Why could it be, in contrast, so difficult to see a relationship between parasitism and *H. defensa*-conferred resistance in natural systems? Rather than whether parasitoid-mediated selection exists, one question might be whether symbiont-conferred resistance is, by itself, strong enough to compensate for the costs of *H. defensa* under field conditions. The most frequently mentioned constitutive costs of *H. defensa* are reduced survival and lowered lifetime reproduction (Zytynska et al., 2021), but the actual extent of the fitness reduction caused by *H. defensa* likely varies in space or time and with biological context: costs depend, for instance, on environmental factors like the host plant (Sochard et al., 2019) and on the combination of aphid genotypes and *H. defensa* strains (Martinez et al., 2018; Vorburger & Gouskov, 2011). Costs can also be of ecological nature and, for example, vary with predator abundance, if resistance to parasitism traded off with resilience to predators as shown in Polin et al. (2014). In our study, we tried to estimate a source of positive selection—parasitism—but not negative selection: we thus ignored that the *net* positive selection acting on *H. defensa* might not be fully proportional to parasitism due to costs which also vary in time. This is one possible reason for the apparently weak relationship between parasitism risk and symbiont frequency we found.

It is well known that the extent of *H. defensa*-conferred resistance depends on the parasitoid species and genotype, in combination with the *H. defensa* strain present in the attacked aphid (Asplen et al., 2014; Cayetano & Vorburger, 2015; McLean & Godfray, 2015; Vorburger et al., 2009). Our results from sequence-typing *H. defensa* in a subset of aphid samples suggest that there was a single dominant strain of *H. defensa* at all our field sites and during both sampling years. We are therefore unlikely to miss relevant changes in the relative frequencies of *H. defensa* strains when looking at overall *H. defensa* frequency. Regarding parasitism risk, however, it is possible that not all parasitoid species and genotypes present at our field sites (Figure 1, Table S5) were equally affected by the presence of *H. defensa*, such that only part of the parasitoids might have selected for *H. defensa*-conferred resistance. The seasonal dynamics of those species or genotypes that are affected by the resistance conferred by the dominant *H. defensa* strain in our data might therefore look different from the dynamics of all parasitoids taken together. The dominance of a single *H. defensa* strain could even facilitate counteradaptation of the parasitoid community to *H. defensa*-conferred protection (Käch et al., 2018; Rossbacher & Vorburger, 2020), such that the mean protective effect might decrease in time.

That *H. defensa* prevalence responded less to parasitism risk than we expected could also be due to an unanticipated characteristic of *H. defensa* offering an additional target for positive or negative selection. Considering the correlation between *H. defensa* frequency and heat days apparent in our data, and the similar findings of Smith et al. (2021) for pea aphids, such a characteristic might be conditional on temperature. Heat can have strong negative effects on aphids (e. g. Asin & Pons, 2001; Ma et al., 2004), and a role in resistance to heat damage is known for other facultative aphid symbionts (e. g. Chen et al., 2000; Montllor et al., 2002), but has also been put up for discussion for *H. defensa* (Russell & Moran, 2006). Endosymbiont-conferred heat tolerance could, for instance, operate through mitigation of negative effects of heat on the obligate symbiont *B. aphidicola* (Burke et al., 2010; Heyworth et al., 2020), or through preparing the host for thermal stress by provoking general stress responses (Brumin et al., 2011). However, heat might also have a negative impact on the ability of *H. defensa* to protect its aphid host against parasitoids (Bensadia et al., 2006; Cayetano & Vorburger, 2013; Doremus et al., 2018; Guay et al., 2009; Higashi et al., 2020). Therefore, the relationship between heat and *H. defensa* could be double-edged: *H. defensa*-infected aphids might benefit from better heat tolerance on the one hand—this should be tested more specifically—but on the other hand, the effect of parasitoid-mediated selection for *H. defensa*-infection could be reduced during heat periods, which would weaken the link between parasitism risk and symbiont prevalence. Generally, the interaction between heat and parasitism, reaching their maximum in the same time period in our survey, might have a different impact on the costs and benefits conferred by *H. defensa* than one would expect based on their individual effects (Heyworth & Ferrari, 2016). Finally, the cost–benefit balance of resistance will also depend on the direct effects of heat on fitness and activity of both aphids and parasitoids (e.g. Parajulee, 2007; Roux et al., 2010).

Smith et al. (2021) propose that not only heat, but also cold might have an influence on *H. defensa* frequencies. This suggestion is based on observing lower symbiont prevalence during colder periods, and more specifically on the drop in *H. defensa* prevalence observed between autumn of one year and spring of the next year. The same trend is seen in Ives et al. (2020) and—although subtly—in our data. This is unlikely due to symbiont losses at the overwintering egg stage, as virtually no losses were observed in hatchlings from eggs of *H. defensa*-infected mothers that were overwintered under artificial as well as natural conditions in a field experiment (Vorburger, Siegrist, & Rhyner, 2017). In contrast to this experiment, we did not sample the very first aphid generation; thus, the observed decrease in *H. defensa* frequency between autumn and spring might not be a direct result from overwintering but rather reflect reduced fitness of *H. defensa*-infected aphids on the primary host plant early in the year, before there starts to be any kind of positive selection for *H. defensa*.

With an overall frequency of 35%, *H. defensa* was by far the most frequent facultative symbiont in the aphid populations we studied, followed by *R. insecticola* with only 8% prevalence (Table S6). The dominance of *H. defensa* and the resulting scarcity of symbiont co-infections in the same aphid reduces the probability that some of the patterns we observe are artefacts of so-called

hitchhiking effects, that is, selection for or against a co-infecting symbiont resulting in unexpected frequency shifts of the focal symbiont (Carpenter et al., 2021). We noted that co-infections between *H. defensa* and *R. insecticola* occurred significantly less than expected by chance, and the same observation has been mentioned multiple times in the past for *A. fabae* (Vorburger & Rouchet, 2016) and *Ac. pisum* (Ferrari et al., 2012; Henry et al., 2013; Mathé-Hubert et al., 2019; Russell et al., 2013; but note the variable results in Rock et al., 2018). Mechanistically, such an effect might come, for instance, from competition between the symbionts or from increased costs experienced by double-infected aphids (e.g. Leclair et al., 2017; but no such effects were found by McLean et al., 2018). Anyway, since the frequency of *R. insecticola* was low and showed no significant temporal dynamics (Table 1, Figure S5), it is unlikely that selection acting on *R. insecticola* had a significant influence on the observed *H. defensa* frequencies. Hitchhiking effects may also be observed if *H. defensa* occurred by chance in a particularly fit or unfit aphid clone, that may have been selected for or against in the course of the season. However, in the genotyped aphid samples from spring and autumn hardly any aphid clone occurred more than once, suggesting that at least on the clone level there were no important frequency shifts that could have interfered with selection for *H. defensa*.

In conclusion, we confirm past studies on *H. defensa* dynamics in stating that short-time parasitoid-mediated selection is more difficult to trace in natural aphid populations than one would expect from experimental evidence. While the repeatedly observed intermediate *H. defensa* frequencies are strongly suggestive of balancing selection, it might be overly simplified to imagine that they are maintained by a simple two-way trade-off between resistance and reproduction. The correlation between *H. defensa* frequencies and heat in our data asks for a more thorough investigation of the role of *H. defensa* under stressful temperatures and adds to the growing evidence that *H. defensa*-conferred benefits may reach beyond protection from parasitoids. Our results underpin how important it is to scrutinize laboratory observations in the field, to gain understanding of the multifarious selection that is acting on defensive symbiosis in the wild.

AUTHOR CONTRIBUTIONS

CV and EG designed the study. EG, JW and CV carried out the field work, JW and EG the laboratory work. EG and CV analysed the data. EG wrote the first draft of the manuscript with inputs from CV and JW, the manuscript was edited and revised by EG and CV. All authors approved the final version for publication.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at https://doi.org/10.5061/dryad.15dv41p2g.

DATA AVAILABILITY STATEMENT

The dataset generated in this study is available at Dryad Digital Repository: https://doi.org/10.5061/dryad.15dv41p2g. The temperature data can be requested directly from MeteoSwiss.

BENEFIT-SHARING STATEMENT

Not applicable.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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