Native parasitoids and a novel invasive host: linking evolutionary ecology and biological pest control
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Chapter 5

Artificial selection for non-reproductive host killing in *Leptopilina heterotoma* for controlling *Drosophila suzukii*

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Abstract

Establishment and spread of invasive species can be facilitated by lack of natural enemies in the invaded area because they might be unable to exploit the invasive pest due to lack of co-evolutionary history. Host-range evolution of natural enemies augments their ability to reduce the impact of the invader and could enhance their value for biological control. We assessed the potential of the larval parasitoid, Leptopilina heterotoma, to exploit the invasive pest Drosophila suzukii. Although this relatively virulent parasitoid has been found to attack and kill the novel host, offspring generally fail to develop. Previously we demonstrated that this native European wasp exhibits significant heritable variation in non-reproductive host killing. In this study, we tested whether killing rate could be improved by artificial selection. We performed seven generations of artificial selection by selecting wasps with the highest killing rate of D. suzukii. Contrary to what we expected, there was a small and inconsistent response to selection in the three selection lines. Realized heritability ($h^2_r$) after four generations of selection was $h^2_r = 0.17$ but was near zero after seven generations of selection. Moreover, a large and consistent positive correlative response was observed with attack rate. We argue that the genetic response might have been masked by an increased D. suzukii fitness due to its adaptation to laboratory conditions. Our study reveals that different steps of the parasitization process need to be considered in the evolution of host-range. It is a proof of principle of how evolutionary principles can be applied to optimize performance of native species for biological control.

Key-words: biological control agents, evolution, genetic improvement, host specificity, host-parasitoid interactions, invasive species, parasitoid, pest control, selective breeding, virulence
1. Introduction

Release from natural enemies can facilitate the invasion of exotic species (Maron & Vilà, 2001; Keane & Crawley, 2002; Colautti et al., 2004). Invasive species can have large detrimental ecological and socio-economic consequences, including harmful effects on agricultural practices (Pejchar & Mooney, 2009; Paini et al., 2016). Escape from natural enemies can arise due to the inability of native predators and parasite species to localize or successfully exploit the invader. For example, native species may not recognize the habitat and/or species-specific cues associated with the invader (DiTommaso & Losey, 2003; Roy, Handley, Schönrogge, Poland, & Purse, 2011). Also, even if native enemies attack a novel species, they may have no or limited impact on the invader. This can occur when the invasive species evolved defence strategies in response to enemies in its area of origin, whereas natural enemies in the invaded area lack a shared evolutionary history impeding the enemy’s fitness (Gandhi & Herms, 2010; Desurmont, Donoghue, Clement, & Agrawal, 2011). The invader can then act as an evolutionary trap, when the preference of a natural enemy for a prey species is disconnected from its performance (Schlaepfer et al., 2002; Schlaepfer et al., 2005; Robertson, Rehage, & Sih, 2013).

Although the immediate performance of native enemies may be inefficient, when genetic variation exists in their ability to exploit the exotic species, evolution might occur towards higher exploitation efficiency (Carlsson et al., 2009). In particular when the invasive (host) species significantly reduces survival and reproduction ability of the native enemy species, it can exert a selection pressure on native enemy species’ traits resulting in either avoidance of the invader or improved detection and exploitation. Although several examples have been documented indicating that evolutionary change can occur even in relative short time (Ashley et al., 2003; Phillips & Shine, 2004; Carroll et al., 2005; Strauss et al., 2006), the frequency of this host-range evolution and its consequences for both the invader and native species are not clear (Strauss et al., 2006; Carlsson et al., 2009). This is, however, important as knowledge of the evolutionary potential of natural enemies in the invasion area aids pest-management programs to mitigate biological invasions and to design strategies for augmentative biological control using already present - native - species (Carroll, 2011; Stotz, Gianoli, & Cahill, 2016; Kruitwagen et al., 2018). A modern strategy in biological pest management is to speed up and direct the evolution of native natural enemies by exploitation of existing intra-specific variation (Lommen et al., 2017; Kruitwagen et al., 2018). Biocontrol agents can be selected and bred with the desired characteristic(s) and then released in the target area (Hoy, 1986; Wajnberg, 2004a; Lommen et al., 2017; Kruitwagen et al., 2018). This method has several advantages compared to traditional biocontrol in particular when releasing exotic biocontrol agents: it mitigates biodiversity risks, reduces non-target effects (De Clercq, Mason, & Babendreier, 2011) and is not hampered by the Nagoya protocol that impedes the import of exotic natural enemies from the pests’ area of origin (Cock et al., 2010; De Clercq et al., 2011; van Lenteren, 2012; Hajek et al., 2016).
We studied the evolutionary potential of the native parasitoid, *Leptopilina heterotoma*, to control the invasive pest species, *Drosophila suzukii*. This fruit fly invaded and has been spreading through Europe and North America since 2008 (Hauser, 2011; Calabria et al., 2012; Fraimout et al., 2017) and has large economic impact on soft fruit production (De Ros et al., 2015; Farnsworth et al., 2017). Most of the investigated native parasitoid species have no or limited impact on the invader because *D. suzukii* has a strong immune response against parasitoids (Kacsoh & Schlenke, 2012; Poyet et al., 2013; Iacovone et al., 2018). It also partly inhabits a different niche compared to their native *Drosophila* hosts in the invaded area (Atallah et al., 2014; Keesey, Knaden, & Hansson, 2015; Karageorgi et al., 2017), which might impair host finding. The relatively highly virulent larval parasitoid *L. heterotoma* has been found to attack *D. suzukii*, but most investigated populations are not able to complete development on this novel host (Chabert et al., 2012; Poyet et al., 2013; Mazzetto et al., 2016; Knoll et al., 2017; Kruitwagen et al., 2021). This indicates a mismatch in host selection behaviour and reproductive performance, and may impede biological control and host-range evolution under natural conditions.

The outcome of parasitization is determined by the progression of stepwise events separated in space and time to pass through different host-defences (Vinson & Iwantsch, 1980; Gross, 1993; Fleury et al., 2009). Parasitization can be divided in host localization, host acceptance, egg laying, and immature development and survival (Kruitwagen et al., 2021). Although *L. heterotoma* is generally unable to reproduce on *D. suzukii*, it can evade some of its defence barriers. It is reported to find the host larvae in the field (Miller, Anfora, Buffington, Daane, Dalton, Hoelmer, Stacconi, et al., 2015) and attempt to exploit them by ovipositor insertion (personal observations) and egg laying (Kacsoh & Schlenke, 2012; Poyet et al., 2013; Stacconi et al., 2015; Iacovone et al., 2018). Interestingly, these behaviours can result in non-reproductive host killing (Chabert et al., 2012; Kacsoh & Schlenke, 2012; Mazzetto et al., 2016; Stacconi et al., 2017; Iacovone et al., 2018; Kruitwagen et al., 2021). This may arise as a result of immune defence costs (encapsulation) of the host (Strand & Pech, 1995; Kraaijeveld, Ferrari, & Godfray, 2002), failure of immature parasitoids to fully develop and emerge (“aborted parasitism” sensu Abram et al. (2019)), mechanical damage due to ovipositor insertion (Samson-Boshuizen et al., 1973), and/or host’ exhaustion of counteracting defences against substances (e.g., venom) injected by the wasp (Rizki & Rizki, 1990; Asgari & Rivers, 2011; Kohyama & Kimura, 2015). Hence, consideration of the stepwise parasitization dynamics may elicit new insights in the formation of host-parasite relationships and their evolutionary potential to exploit novel hosts (Agrawal & Lively, 2003; Elena & Lenski, 2003; Duneau, Luijckx, Ben-Ami, Laforsch, & Ebert, 2011; Hall, Bento, & Ebert, 2017; Kaser et al., 2018).

Traits underlying non-reproductive host mortality in parasitoid systems can in part be genetically determined and therefore be subject to evolutionary change (Henter, 1995; Kraaijeveld, Hutcheson, Limentani, & Godfray, 2001; Henry et al., 2008; Henry et al., 2010; Colinet et al., 2013; Cavigliasso et al., 2019; Mathe-Hubert et al., 2019). However, whereas most research focusses on the main outcome of host-parasitoid interactions, i.e. reproductive success, little is known about consequences of the previous steps, such as host-killing, for population control and evolution of host-parasitoid interactions (Abram et
al., 2019). Presence of genetic variation in both reproductive and non-reproductive traits could be exploited for artificial selection to improve biological control efficacy (Kruitwagen et al., 2018). Also, non-reproductive effects might drive adaptive processes (e.g., formation of novel biotic interactions) when traits that determine host-killing are positively correlated with reproductive success. Alternatively, a negative relationship would constrain adaptation and might endanger population persistence and/or promote selection for host-range conservation.

In this study, we investigate the potential of the native parasitoid *L. heterotoma* to adapt to the novel highly resistant host *D. suzukii*. Our previous study (this thesis Chapter 3) revealed significant heritable variation in attacking and killing the novel host in a European outbred population. Here, we test the hypothesis that artificial selection can increase non-reproductive effects. We also investigate correlated responses of other steps in the parasitization behaviour, such as attack rate and reproductive success. Finally, we test whether wasp offspring that successfully developed on *D. suzukii* have increased reproductive success on *D. suzukii* in subsequent generations.

2. Material and methods

Parasitoid and *Drosophila* lines

A genetically diverse strain of the parasitoid *Leptopilina heterotoma* was established from crossing individuals of seven European populations (two populations from Spain, two from the Netherlands and three from France) (this thesis Chapter 3). It was maintained on a relatively low-resistant *Drosophila melanogaster* host strain (WW) at 25°C, under a light-dark regime of 16:8. These flies were derived from wild flies collected near Leiden, the Netherlands, received in 2009, and kept as mass cultures at 20°C in quarter pint bottles containing 30 ml medium (agar (17g/L), yeast (26g/L), sugar (54g/L) and nipagine (16,7 ml/L)). For artificial selection a *D. suzukii* was used collected from Westland, the Netherlands in 2016. It was reared in quarter pint bottles containing 30 ml cornmeal diet (agar (10 g/L), glucose (30 g/L), sucrose (15 g/L), heat-inactivated yeast (35 g/L), cornmeal (15 g/L), wheat germ (10 g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid (5 mL/L), and Tegosept (2 g/L)).

Standardized parasitization performance measurements

Individual parasitization performances were measured following a standardized parasitization performance test described in Chapter 3. In short, each female was placed in a vial with 25 *D. suzukii* larvae for four hours to parasitize. The number of emerging *D. suzukii* flies that survived wasp exposure and the number of wasp offspring were counted. Moreover, flies that emerged were inspected under the microscope for presence of at least one encapsulated parasitoid egg to quantify the number of hosts that had been parasitized but successfully mounted an immune response (encapsulation). Control vials were maintained on each testing day to measure host survival in absence of a parasitoid. Each wasps’ killing rate was then quantified as the percentage of flies killed in excess to the
mortality of non-exposed flies. The attack rate was the percentage of flies that were parasitized, as estimated from the excess mortality in the larvae due to wasp exposure and the number of flies that were attacked but survived (i.e., flies that successfully mounted an immune response (encapsulation). Encapsulation of wasp eggs by the host was quantified by squashing the flies between two object glasses and inspection under the microscope for presence of a melanized egg. As measure of reproductive success, the successful parasitism was calculated as the proportion of flies killed that yielded wasp offspring.

**Artificial selection for killing rate**

Wasps from the genetically diverse population (P) were randomly divided over six lines: three selection (S) and three control lines (C), each consisting of 100 males and 100 females. Due to the time-intensive nature of setting up each generation and the artificial selection procedure, this was the largest possible population size that allowed for imposing a selection regime while minimizing influences of inbreeding, genetic drift and rapid depletion of genetic diversity (Weber & Diggins, 1990; Fry, 2003). Control lines were kept to investigate whether any change over time was due to other causes rather than response to selection on killing. The three replicate selection lines were set up to distinguish selection from drift, as consistent changes in the same direction applied across all replicate lines relative to the unselected control lines is unlikely to be due to drift, as drift acts in a random manner. Moreover, we estimated the realized heritability ($h^2_r$), the response to selection as proportional to the amount of selection applied, to quantify the degree of phenotypic change due to selection (see below) (Falconer & Mackay, 1996; Lynch & Walsh, 1998). Each line was selected for seven successive generations (F1 – F7).

Each generation, phenotypic variation was quantified within each replicate line after which the best performing individuals were selected and cultured. Killing rate was measured of each individual female following the standardized performance test, and the 50 (out of 100) females with the highest trait value were chosen to contribute to the next generation. Besides selection for killing rate, attack rate and reproductive success were measured to investigate potential correlated response to selection. Due to logistics, individuals from each line were tested over a period of 2-5 days. Selection of 50% of the individuals was chosen to reduce inbreeding, genetic drift and chance of rapid depletion of genetic diversity. In particular, as host killing is a trait potentially controlled by many genes, strong selection of a small proportion could increase the chance of losing beneficial alleles and thus deplete genetic diversity and reduce response to selection.

Parasitization of *D. suzukii* during the performance tests rarely yielded wasp offspring. Therefore, the highest performing mothers were placed individually in vials on the low resistant host, *D. melanogaster*, to generate the next generation. Offspring were collected by taking eight random parasitoid pupae from each of the mothers just before emergence, and these were then divided over two agar bottles (one served as back-up). This allowed the offspring to mate among each other and thus reduced chances of sib-mating. Offspring were kept in the agar bottles at 20°C until the performance of 100 randomly chosen females was measured and compared again (hence each line always remained at the constant size of 100 females). The same protocol was followed for the control lines except that each
generation 50 random females were chosen to contribute to the next generation. Moreover, as phenotypic evaluations are labour intensive, killing performance of control lines could only be tested and compared to the selection lines within generations 5 and 7 of artificial selection.

**Repeated selection on reproductive success**

During artificial selection on killing rate, parasitoids were occasionally able to successfully reproduce on *D. suzukii*. To test whether offspring that emerged from *D. suzukii* differed in *D. suzukii* exploitation performances and whether this could increase offspring developmental success, a separate selection line was created from *D. suzukii* reproducers (R1) alongside the three artificial selection and control lines, and subjected to selection for reproductive success. Offspring that emerged during each generation of the selective breeding on killing rate were added to the R1 and used as starting material for selection on reproductive success. Females of the R1 were tested following the standardized performance test, and offspring that emerged from *D. suzukii* were selected and used to set up a new selection line (R2). Females of the R2 line were then again tested and offspring that emerged from *D. suzukii* were collected and used to set up a third line, R3. Note that the R1 wasps were bred alongside the selection on killing rate and after each round of selection (P-F7) on killing rate new genetic material was added to the R1 and allowed to mate *D. suzukii* reproducers. We therefore decided to test and select the R1 on reproductive success each generation after new parasitoids were added to the line, resulting in a repeated selection process of the same line but each time with the novel added genotypes. The R1 was subjected to eight selection rounds. Similarly, the R2 was bred simultaneously with the R1, and was also repeatedly tested and selected as new genetic material was added to the population from the R1 (in total 8 times selected). To test whether the line selected for repeated reproductive success on *D. suzukii*, R3, differed in killing rate and reproductive success, the R3 line was tested and compared to the base population (P) which was used as starting point of selection and the selection line that best responded to selection on host-killing (S2), following the individual exploitation performance test.

**Statistical analysis**

Fly survival and parasitization performance indices were analysed with generalized mixed models for binomial data by specifying a two-column matrix with the number of “successes” and “failures” using the lme4 package (Bates et al., 2014). The response to selection was analysed by fitting fly survival as dependent variable and treatment (wasp presence/absence), generation (as continuous) and their interaction as fixed factors, and date and line as random factors. Parasitization performances between wasp lines were compared by correcting for day-to-day variation in fly survival. To this end, performances were standardized with the average fly survival of the control flies that were not exposed to wasps on the same testing day. We tested for overdispersion by comparing the sum of squared Pearson residuals to the residual degrees of freedom following Bolker et al. ([http://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#overdispersion](http://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#overdispersion)). When overdispersion was detected (α = 0.05), observation level random effect was added by giving each data point a random effect with a unique level (Harrison, 2015). Significance of
main effects was tested by comparing the full model to the model without the fixed effect by ANOVA. Post-hoc comparison of means was performed with Tukey tests, using the emmeans package (Lenth, 2020).

**Realized heritability during artificial selection**

The response to selection also provides an estimate of realized heritability ($h^2_r$) of killing rate, which can be estimated as the slope from the linear regression of the cumulative response to selection over the cumulative selection differential forced to the origin (Falconer & Mackay, 1996). For each replicate line, we calculated the response to selection ($R$) as the mean offspring value minus the mean of the total parental population, and the selection differential ($S$) as the mean of the selected parents minus the mean of the total parental population. Phenotypic values were calculated as the percentage of flies killed standardized for the average fly survival of flies not exposed to wasps.

### 3. Results

#### Response to selection: killing rate

Fly survival in absence and presence of wasps was variable within and between generations, but overall fly survival was significantly reduced when exposed to parasitoids (Fig. 1a) (GLMM, $\beta = -0.71$, $\chi^2(1) = 122.38$, $p<0.01$). The average *D. suzukii* survival in absence of parasitoids was relatively constant during the first two generations, but increased from the F2 onwards. The average survival rate in the F7 in the absence of wasps was 30% higher compared to the F2. This trend was supported by observed changes in fly rearing quality: i.e. increased fecundity and survival. The percentage of flies that parasitoids were able to kill initially ranged from 0 to 69.3% with an average of 24.0% ± 0.96SE (Fig. 1b). During the first four generations of selection, the average killing rate increased to 36.1% ± 1.27 SE (ranging from 0 to 100%) and the percentage of individuals that killed > 40% flies increased from 17% to 43%. During the last three generations average killing performance and the number of wasps with killing performance of >40% decreased however to 20.7% ± 0.8SE and 10% respectively (F7). The increased fly survival and the decrease in killing performance after the F4 generation suggests that fly host fitness increased over time. This hampers comparison of between generation performances of the wasps, as the trait value of killing performance is partly influenced by the fitness of the host.

The response to selection was therefore investigated by comparing the slope between fly survival and generation in presence and absence of parasitoids (Fig. 1). GLMM analysis indicated that the temporal trend in fly survival was best predicted by fitting ‘generation’ as quadratic variable (linear vs. quadratic: $\chi^2 = 5.00$, $p<0.01$). The estimates of the regression lines confirmed that parasitoids significantly reduced fly survival ($\beta = -0.68$, SE=0.04, $p<0.01$), and fly survival (as quadratic term) increased over generations independent of
treatment ($\beta = 0.01$, SE=0.002, $p<0.01$). There was however also a significant interaction between treatment (wasp presence/absence) and generation (GLMM, change in slope in presence versus absence of wasps, $\beta = -0.004$, SE=0.001, $\chi^2(1)=11.53$, $p<0.01$), which reflects that fly survival in absence of wasps increased relatively more than in the presence of selected wasps. In other words, the proportion of flies killed increased slightly despite the improved survival rate of the host, and thus confirms an effect of selection.

**Selected vs unselected lines:** After five generations of selection, wasps from the Selection lines killed significantly more flies compared to those from Control lines (Fig. 2a) (GLMM $\chi^2(1)=4.08$, $p=0.04$). Analysis of each replicate line separately revealed that selected wasps
of two replicate lines (S2 and S3) had a higher killing rate (GLMM line2: $\chi^2(1) = 6.21$, $p=0.01$; line3: $\chi^2(1) = 5.52$, $p=0.02$) and the other replicate (S1) performed similarly to its unselected control line (Fig. 2b) (GLMM, $\chi^2(1) = 1.29$, $p=0.25$). Similarly, after two more rounds of selection (F7), selected wasps killed significantly more flies compared to unselected wasps (Fig. 2c) GLMM $\chi^2(1) = 5.57$, $p=0.02$). Analysis of each replicate line separately demonstrated that selected wasps of two lines (S1, S2) (almost) significantly reduced $D$. suzukii survival relative to their unselected controls (GLMM line1: $\chi^2(1) = 4.89$, $p=0.03$; line2: $\chi^2(1) = 3.7$, $p=0.054$), but one line (S3) did not differ in killing performance (Fig. 2d) (GLMM, $\chi^2(1) = 0.004$, $p=0.95$). Yet, whereas selected and control wasps in the F5 generation had maximum trait values up to 100%, the highest killing rate in generation F7 was 63%, and the average killing rate had decreased with 2%. Taken together, selected wasps had increased killing rate compared to non-selected individuals, but the differences were relatively small. The decrease in host killing ability is in line with our observed increased host fitness, which reduced the response to selection. Unfortunately, due to logistic constraints, we were only able to measure and compare the unselected control wasp lines at generation 5 and 7.

Figure 2 | Killing rate of selected and unselected *L. heterotoma* wasps after five (A, B) and seven (C, D) generations of artificial selection, of the three replicate lines combined (n=300) (A,C) and of each replicate line separately (n=100) (B,D). Killing rate was calculated as the percentage of flies killed, adjusted for non-exposed fly mortality. Horizontal lines represent median killing rate and inner thick vertical line the interquartile range. Statistical differences by Tukey’s post hoc test.
Realized heritability

Realized heritability ($h^2_r$) estimates (i.e. the response to selection as proportion of the amount of selection applied) fluctuated from zero to 0.310 between generations during the artificial selection experiment, but was not significantly different from zero after seven generations ($p>0.05$) (Fig. 3, Table 1). The average heritability was largest over the interval from P to F4, $h^2_r = 0.167$, and differed significantly from zero ($p=0.01$) (Fig. 3, Table 1). Replicate lines also differed in response, the heritability estimate did significantly differ from zero for replicate line 3 after three generations of selection ($h^2_r = 0.31$, $p<0.05$), but not for the other replicates for any interval.

![Figure 3](image.png)  

Figure 3 | Realized heritability ($h^2$) of killing rate in artificial selection experiment of *L. heterotoma*. Cumulative response to selection (R) is plotted as function of the cumulative selection differential (S) for each replicate selected on increased killing rate for seven generations (dashed lines). The average realized heritability ($h^2$) was calculated as the slope of the linear regression between R and S until generation four and seven (straight lines). The grey area represents the cumulative response to selection until generation 4.
Table 1 | Realized heritability estimates ($h^2_r$) for killing rate calculated as the slope of the linear regression between cumulative selection differential and response to selection over different generation intervals. Asterisk indicates whether the slope of the regression significantly differed from zero (p<0.05), and bold indicate the highest heritability values of each replicate line.

<table>
<thead>
<tr>
<th></th>
<th>Realized heritability (std. error)</th>
<th>F-statistic</th>
<th>adj. R$^2$</th>
<th>p-value</th>
</tr>
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<tbody>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-F3</td>
<td>0.143 (0.081)</td>
<td>$F_{1,8}=3.21$</td>
<td>0.1973</td>
<td>0.111</td>
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<tr>
<td>P-F4</td>
<td><strong>0.167 (0.053)</strong> *</td>
<td>$F_{1,11}=9.69$</td>
<td>0.420</td>
<td>0.010</td>
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<tr>
<td>P-F5</td>
<td>0.033 (0.052)</td>
<td>$F_{1,14}=0.40$</td>
<td>-0.041</td>
<td>0.536</td>
</tr>
<tr>
<td>P-F6</td>
<td>0.017 (0.036)</td>
<td>$F_{1,17}=0.45$</td>
<td>-0.046</td>
<td>0.648</td>
</tr>
<tr>
<td>P-F7</td>
<td>-0.001 (0.027)</td>
<td>$F_{1,20}=0.001$</td>
<td>-0.050</td>
<td>0.961</td>
</tr>
<tr>
<td>Replicate 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-F3</td>
<td>-0.036 (0.036)</td>
<td>$F_{1,2}=0.96$</td>
<td>-0.012</td>
<td>0.430</td>
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<tr>
<td>P-F4</td>
<td><strong>0.074 (0.053)</strong></td>
<td>$F_{1,3}=1.89$</td>
<td>0.182</td>
<td>0.263</td>
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<tr>
<td>P-F5</td>
<td>0.013 (0.045)</td>
<td>$F_{1,4}=0.09$</td>
<td>-0.223</td>
<td>0.782</td>
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<tr>
<td>P-F6</td>
<td>-0.007 (0.032)</td>
<td>$F_{1,5}=0.05$</td>
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<td>0.838</td>
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<tr>
<td>P-F7</td>
<td>-0.014 (0.023)</td>
<td>$F_{1,6}=0.36$</td>
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<td>0.572</td>
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<tr>
<td>Replicate 2</td>
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<tr>
<td>P-F3</td>
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<tr>
<td>P-F4</td>
<td><strong>0.246 (0.137)</strong></td>
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<tr>
<td>P-F5</td>
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<td>0.921</td>
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<td>P-F7</td>
<td>-0.005 (0.071)</td>
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<td>-0.165</td>
<td>0.941</td>
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<tr>
<td>Replicate 3</td>
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<tr>
<td>P-F3</td>
<td><strong>0.310 (0.059)</strong> *</td>
<td>$F_{1,2}=27.30$</td>
<td>0.897</td>
<td>0.035</td>
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<tr>
<td>P-F4</td>
<td>0.162 (0.073)</td>
<td>$F_{1,3}=4.86$</td>
<td>0.491</td>
<td>0.115</td>
</tr>
<tr>
<td>P-F5</td>
<td>0.060 (0.071)</td>
<td>$F_{1,4}=0.71$</td>
<td>-0.062</td>
<td>0.447</td>
</tr>
<tr>
<td>P-F6</td>
<td>0.035 (0.052)</td>
<td>$F_{1,5}=0.45$</td>
<td>-0.102</td>
<td>0.534</td>
</tr>
<tr>
<td>P-F7</td>
<td>0.011 (0.041)</td>
<td>$F_{1,6}=0.08$</td>
<td>-0.151</td>
<td>0.790</td>
</tr>
</tbody>
</table>

**Correlated responses to selection**

**Attack rate**

Attack rate (i.e. the percentage of flies killed plus flies that survived but with encapsulated wasp egg) was calculated from a random sample (n=30) of each replicate line at the start of selection (P) and in generations F4, F5 and F7. Wasps successfully attacked 64.5% ± 1.47 SE flies (all data combined), and 37.0% ± 1.50 SE of the attacked flies were killed (i.e. lethal attack rate). There was a near significant systematic increase in attack rate over time (Fig. 4a) (GLMM, $\beta=0.06$, SE=0.035, $\chi^2(1)=3.68$, p=0.051) and generation had a significant effect when taken as categorial variable (GLMM, $\chi^2(3)=17.20$, p<0.001). The percentage of attacked flies was highest in the F4 generation compared to unselected wasps at the start (P) and the F5 and F7 generations (Tukey’s test, p<0.001). Generation did not have a significant effect on the proportion of attacked flies that were killed when taken as
continuous explanatory variable (GLMM, $\chi^2(1) = 2.94$, $p=0.09$) nor as categorical variable (GLMM, $\chi^2(3) = 6.59$, $p=0.09$).

There was no difference in attack rate between selected and unselected wasps in the F5 generation (Fig. 5a) (GLMM, $\chi^2(1) = 1.57$, $p=0.21$), neither in lethal attack rate (Control lines: $30.0\% \pm 30.2$ SE, Selection lines: $30.6\% \pm 27.8$, GLMM, $\chi^2(1) = 1.07$, $p=0.30$). However, after seven generations of selection (F7), selected wasps had a significantly higher attack rate (GLMM, $\chi^2(1) = 12.41$, $p<0.001$), though the proportion of killed flies was again similar (Control lines: $34.7\% \pm 29.5$ SE, Selection lines: $29.6\% \pm 21.6$ SE, GLMM, $\chi^2(1) = 0.29$, $p=0.59$). Selection on killing performance thus seemed to have resulted in an increased attack rate, but wasps did not become more efficient in host killing as there was no clear effect on lethal attack rate. Note, however, that attack efficiency could also be confounded by the increased host fitness over the course of the experiment.

**Reproductive success**

Taking all generations into account, 13% (273/2381) of the wasps produced at least one offspring in *D. suzukii*. The majority, 82.8%, produced one offspring (n=226) in a batch of 25 larvae, and only 12% produced two (n=34), 4% three (n=10), 0.7% four (n=2) and one

Figure 4 | Correlated responses to selection for killing rate in *L. heterotoma*: (A) attack rate and (B) successful parasitism averaged over the three selection lines (dark blue), and of each selection separately (light blue dots). The average attack rate was calculated as the percentage of flies killed plus flies that survived but with encapsulated wasp egg. Successful parasitism was calculated as the percentage of killed flies that yielded offspring. Error bars represents standard errors of the mean. Note that sample size of the attack rate in the F4 was reduced (n=60), as collected flies from one selection line were lost.
female produced five offspring. We tested whether killing rate was related to reproductive success, i.e. whether those wasps able to reproduce had a higher killing activity compared to those that were not able to reproduce. Killing rate was compared between individuals that killed at least one host that resulted in one parasitoid offspring (n=266), to those that also killed at least one host (killing rate >0), but did not reproduce (n=1652). This revealed no difference in killing performance between wasps that were able to reproduce and those that were not (32.8% ± 1.19 SE, 31.2% ± 0.48 SE respectively, GLMM, $\chi^2 (1) =0.583$, $p=0.45$).

Successful parasitism (i.e. the percentage of killed flies that yielded wasp offspring) differed between generations (Fig. 4b) (GLMM, $\chi^2 (7) =26.06$, $p<0.001$): it was highest in the F3 and F4 generations compared to first generations P-F2 and last generations F5- F7. Reproductive success in the first two generations (P-F2) and last two generations (F5-F7) was quite similar. This could correspond with the unfit host conditions during the first generations thereby decreasing their “quality” to support offspring survival, and the increased host fitness and thereby immune resistance in the latter generations. Comparison of selected and unselected wasps demonstrated that unselected wasps had a larger reproductive success in the F5 generation. There were slightly more individuals able to reproduce (Control lines: 13.1%, Selection lines: 8.3%, GLMM, $\chi^2 (1) =3.64$, $p=0.056$), and unselected wasps had a significant higher successful parasitism rate (6.3%) compared to selected ones (3.1%) (Fig. 5b) (GLMM, $\chi^2 (1) =4.85$, $p=0.03$). After seven generations of selection the number of wasps that successfully reproduced did not differ between selected (10.9%) and unselected wasps (9.1%) (GLMM, $\chi^2 (1) =0.52$, $p=0.47$). Also, successful parasitism did not differ between selected and unselected wasp lines (C: 2.9%, S: 3.0%, GLMM, $\chi^2 (1) =0.012$, $p=0.91$). Mechanisms of increased host killing ability thus do not seem to increase the probability of offspring survival. In addition, reproductive success seems largely determined by host fitness as indicated by significant differences between generations in relation to fly fitness.

**Repeated selection on reproductive success**

Over the course of the eight generations (P-F7) of selective breeding for host-killing, wasp offspring that emerged from *D. suzukii* were collected and used to set up a new line (Reproducers, or R1). In total 102 females were collected that successfully emerged from *D. suzukii* during the course of the experiment. The performance tests of R1 females on *D. suzukii* resulted in 41 female offspring (R2). Selection of the second generation of successful reproducers resulted in 11 females that emerged from *D. suzukii* (R3). Parasitization performances of the third generation of reproducers (R3) were compared to the genetically diverse stock population (P), replicate 2 of the selection on killing (S2) - as this line showed the largest and most consistent response to selection - and its control line (C2) (n=25). Overall, parasitoids reduced fly survival (GLMM, $\beta =-0.84$, $\chi^2 (1) =10.68$, $p<0.001$), but parasitoids with a history of developing on *D. suzukii* did not have higher killing rate (GLMM, $\chi^2 (3) =6.65$, $p=0.08$). In fact, the R3 and the unselected wasp line (C2) killed slightly fewer flies compared to the wasp line selected for host killing (S2) and the diverse stock population (P) (R3: 16.7% ± 3.48 SE, C2: 15.4% ± 2.55 SE, S2: 22.9% ± 2.57
SE, P: 21.9% ± 2.53 SE). None of the wasps were able to reproduce on *D. suzukii*. Hence wasps selected on host killing and reproductive success did not differ in reproductive success on *D. suzukii*.

Figure 5 | Correlated response to selection for killing rate in *L. heterotoma*. Attack rate (A) and Reproductive success (B) of selected and unselected wasps after five (F5) and seven (F7) generations of selection on killing performance. Attack rate was calculated as the percentage of flies killed, corrected for mortality in non-exposed flies, plus those that were parasitized but survived as measured from encapsulated parasitoid eggs (n=90). Reproductive success was calculated as the percentage of killed flies that yielded offspring (n=300). Horizontal lines represent median killing performance and inner thick vertical line the interquartile range.

4. Discussion

We investigated whether the invasive pest *D. suzukii* can become a novel host for the native parasitoid *L. heterotoma* by focussing on three sequential steps of the parasitization process: (1) attack rate (2) host killing and (3) successful offspring development (reproductive success). Previously we showed that *L. heterotoma* exhibits significant heritable variation in attack rate ($h^2 = 0.44$) and host killing ($h^2 = 0.28$), but not in offspring survival ($h^2 = 0.0$) (this thesis Chapter 3). However, contrary to our expectations, the response to selection after seven generations of directional selection on increased killing rate relative to the amount of selection applied, i.e. the realized heritability ($h^2_r$), was zero. Moreover, selection yielded increased killing rate in selected wasps compared to non-selected individuals at generation five and seven, but the differences were relatively small (2 to 8%) and were inconsistent among replicates, indicating that these differences could be due to either selection or drift. Yet, realized heritability after the first four generations was significant, $h^2_r = 0.17$, and similar to our previous heritability estimate in half-sib analysis (this thesis Chapter 3). Additionally, we did find a consistent and strong correlative response in the attack rate (15% improvement relative to control lines). The
decline in response to selection is in line with our observed increase in *D. suzukii* fitness, which might have reduced the response to selection. Selection did not improve reproductive success and parasitoid offspring that emerged from *D. suzukii* did not exhibit enhanced killing and reproduction, suggesting that the ability to overcome host defences did not directly affect the success rate at the intermediate steps in the parasitization process.

**Factors that reduce response to selection**

The increase in host-killing rate in response to artificial selection was minor. Several factors could have reduced the magnitude of response to selection. An important factor is variation in phenotypic traits of the host: the likelihood of being found and surviving parasitoid attack (Kruitwagen et al., 2018). Given that over the course of the selection experiment (one year) *D. suzukii* survival increased with 30% and the stock population had only been established several months before initiation of the experiment, laboratory adaptation in the host strain seems to have occurred. Following the resource allocation theory, resources invested in life history traits such as growth and reproduction can come at the expense of other energetic costly traits, like the maintenance and deployment of immune defences (Rauw, 2012; Schwenke, Lazzaro, & Wolfner, 2016). These life-history trade-offs can become more pronounced under environmental stress, such as food limitation and desiccation (Moret & Schmid-Hempel, 2000; Hoang, 2001). Hence, when the *D. suzukii* stock population became more adapted to the laboratory, more resources might have become available to allocate to immunity, making the flies more resistant to parasitoid attack. The low improvement in killing performance after seven generations of selection could then be explained by the relative faster evolution of the hosts compared to the parasitoids’ killing rate. This slowed down the response to selection, albeit it was still detectable, and even decreased the wasps’ ability to kill the flies despite evolution of killing rate. Alternatively, *D. suzukii* might have evolved specific counter defences to *L. heterotoma* over the course of the experiment. However, flies that survived parasitoid attack were never placed back in the stock population, making evolution of resistance to parasitoid attack highly unlikely.

Besides the increased *D. suzukii* host fitness, several other genetic and environmental factors could have contributed to the low response to selection. Firstly, being a complex behavioural trait, killing performance likely depends on many genes. For example, virus-like particles (Poirié, Carton, & Dubuffet, 2009; Cavigliasso et al., 2019; Mathe-Hubert et al., 2019), ovipositor morphology (van Lenteren, Isidoro, & Bin, 1998) and neuro-sensory characteristics (van Lenteren et al., 2007; Ruschioni, van Lenteren, Smid, & van Lenteren, 2015) have been shown to be important for parasitization in *L. heterotoma* and might be subject to evolutionary change. Therefore, it would take multiple generations to establish a shift in allele frequencies (Falconer & Mackay, 1996). Secondly, evolutionary change might have been limited due to a decrease in additive genetic variance and/or loss of alleles due to genetic drift over successive generations of selection. However, this is assumed to become more pronounced in long term selection experiments and under strong selection or bottlenecks (Falconer & Mackay, 1996; Barton & Partridge, 2000; Careau, Wolak, Carter, & Garland Jr, 2013). Thirdly, our estimate of heritability of killing rate in the base
population was $h^2 = 0.28$ (this thesis Chapter 3), meaning that phenotypic variance is also influenced for a substantial part by non-additive genetic variation, reducing the potential for selection. Similarly, Henter (1995), estimated that variation in successful parasitism rate was attributed for 57% to environmental effects in the aphid parasitoid *Aphidius ervi*. Natal host quality (*D. melanogaster*) (this thesis Chapter 4; Rosenheim & Rosen, 1992; Harvey, 2000; Ris, Allemand, Fouillet, & Fleury, 2004), and the wasps’ nutritional status (Ellers et al., 1998; Jervis, Ellers, & Harvey, 2008) could also have influenced the wasp’s ability and willingness to parasitize. Although every attempt was made to maintain constant conditions, we cannot exclude any unintentional environmental variation. This underlines the importance of understanding the sources of variation to increase the response to selection when attempting to genetically improve natural enemies for biocontrol (Kruitwagen et al., 2018).

**Mechanisms underlying the evolution of non-reproductive host mortality**

Our findings are in line with previous reports on the widespread nature of non-reproductive host mortality in host-parasitoid systems (Heimpel, Neuhauser, & Hoogendoorn, 2003; Abram et al., 2016; Abram et al., 2019), including the *L. heterotoma – D. suzukii* system (Chabert et al., 2012; Kacsoh & Schlenke, 2012; Mazzetto et al., 2016; Iacovone et al., 2018). The larvae of koinobiont parasitoids, like *L. heterotoma*, feed from the developing host larvae until the parasitoid reaches the pupal stage, after which the host dies (Fleury et al., 2009). However, when the host dies too soon the parasitoid is unable to develop and survive (Rizki & Rizki, 1990). This raises the question which mechanism(s) underlie the induction of non-reproductive host mortality. Generalist species like *L. heterotoma* might have an “opportunistic” strategy reflected in their low threshold for host species acceptance, readily parasitizing novel hosts like *D. suzukii*, combined with a high egg load (i.e. eggs are less ‘costly’). Alternatively, they might be unable to distinguish between host (habitat) cues of suitable hosts and *D. suzukii* that predict offspring survival. Consequently, non-reproductive host mortality might be a ‘by-product’ of selection for parasitizing on other hosts. Evolution of non-reproductive host-killing and the evolutionary consequences of these maladaptive host choices have not been studied extensively. Our findings provide new avenues for such efforts to understand and predict the evolution of this trait and how this influences host range evolution (this thesis Chapter 6).

Interestingly, selection on host killing resulted in a correlated response in attack rate, but wasps did not become more efficient in host killing. Perhaps wasps increased their search time, acceptance rate and/or ability to recognize hosts’ species presence as a result of selection. An increase in lethal attack rate could then have come about by not only attacking more hosts, but also by attacking the same host multiple times and thus further enhancing their damage. Activity level in *L. heterotoma* (Fleury, Allemand, Fouillet, & Boulétreau, 1995) and host selection in the *A. tabida – Drosophila* system (Mollema, 1990; Rolff & Kraaijeveld, 2001), were also found to be partly determined by genetic effects, suggesting that these first steps of parasitization process (host finding – acceptance) can evolve relative quickly in response to selection. The lack of response in lethal attack rate in our experiment was surprising as European populations expressed major differences (this
thesis Chapter 3). Moreover, lethal attack rate had a relatively high heritability $h^2 = 0.61$ (this thesis Chapter 3). Considering that the lethal attack rate was slightly higher in the F4 compared to the start of the experiment, but then decreased in the F5 and F7, this trait is likely more sensitive to the rise in fitness of D. suzukii than the attack rate. As a consequence, such a difference in environmental sensitivity of different parasitization parameters can facilitate maintenance of variation for the outcome of host-parasite interactions (Duneau et al., 2011; Ebert et al., 2016; Hall et al., 2017). More research is needed to identify specific ‘traits’ underlying host killing and their sources of variation to understand what could constrain or favour formation of novel host-parasitoid associations.

**Host killing as intermediate step to a complete host shift/expansion**

The relationship between host choice and offspring survival is fundamental for parasitoids’ fitness, host-range and population persistence (Jaenike, 1978; Thompson, 1988; Henry et al., 2005). Maladaptive host choices, that is attacking hosts that do not support offspring development, can impose costs (e.g., time and resources) on the individual and lead to an evolutionary trap when females are not able to discriminate between suitable and unsuitable host species (Schlaepfer et al., 2005; Abram, Gariepy, Boivin, & Brodeur, 2014; Abram et al., 2019). We therefore investigated the severity of the D. suzukii ‘trap’ for L. heterotoma to see whether females are able to overcome the host defensive barrier. We found however no positive relationship between host-killing and reproductive success and no indications of genetic variation in reproductive success. This suggest that host-killing is not an effective step to a complete host-shift/expansion in the short term in this experimental system. Although we did not find indications of host-shift/host-range expansion evolution, this might occur when a genetic variant appears that is able to evade host-immunity and survive inside the host. Future sampling and testing of populations in space and time might reveal how natural enemies evolve to the novel host: i.e., whether a novel biotic interaction arises, or alternatively, whether they evolve to avoid this host.

The evolution of a generalist strategy can be restricted by trade-offs: adaptation to one resource can decrease fitness in another (Fry, 1990; Jaenike, 1990; Agrawal, 2000; Via & Hawthrone, 2002; Elena & Lenski, 2003; Henry et al., 2008). In order to reproduce on D. suzukii, parasitoids might need another parasitization strategy then on their native hosts like D. melanogaster. As such, a complete host shift might have been hampered in our selection experiment as parasitoids were cultured on D. melanogaster. Interestingly, unselected wasps showed a slightly larger reproductive success in the F5 compared to selected ones and selection on reproductive success did not enhance either killing or reproductive success rates. So, evolution of host-range might also have been constrained by functional correlations between traits within the same host: the parasitoids’ host-killing mechanism might not always promote offspring survival. For example, superparasitism (i.e., laying multiple eggs in the same host) or high venom quantity might kill the host before full development of the parasitoids. Selection, however, did not seem to affect exploitation success when the wasps were provided with D. melanogaster as there was no large change observed in reproductive success during and after selective breeding (>70% successful parasitism) and comparison of selected and control lines in the F7 (n=65) yielded no
significant differences in killing rate of *D. melanogaster* (GLMM, $\chi^2 (1) = 0.807$, $p = 0.37$) and successful parasitism (GLMM, $\chi^2 (1) = 0.807$, $p = 0.1$).

**Implications of non-reproductive host-killing for biocontrol**

Despite the widespread nature of non-reproductive effects in host-parasitoid systems, they are underappreciated for biocontrol, as the main focus is often on reproductive success (Abram et al., 2016; Abram et al., 2019). Recent findings, however, indicate that these effects can be an important mechanism that augment the biocontrol performance of parasitoids (Münster-Swendsen, 2002a; Huang, Hua, Wang, Zhang, & Li, 2017a; Kaser et al., 2018). Our study adds that non-reproductive host killing has a genetic component, but is also sensitive to environmental conditions resulting in fluctuating heritability. Presence of significant heritable variation thus does not guarantee improvement by artificial selection, this can only occur when stable environmental conditions (e.g., host fitness) over the course of selection and high accuracy of selection can be achieved. For example, if environmental conditions, intensity of selection and heritability would have remained constant over generations in our experiment, following the breeders’ equation ($R = \frac{1}{t} \sigma_a \sqrt{h^2}$) (Falconer & Mackay, 1996), it would have taken about seven generations to increase killing rate to 97.25% (assuming selection intensity $(i)$ of 0.79 (Wricke & Weber, 1986), additive genetic variance of $\sigma_a^2 = \sqrt{1.08}$ and $h^2 = 0.28$ (this thesis Chapter 3)).

To understand and predict the ability of *L. heterotoma* to regulate *D. suzukii* population growth under field conditions and agricultural settings (e.g., greenhouses or orchards), more research is needed in parasitoids’ ability to find hosts and it's host species preference. In addition, following our observation that *D. suzukii* resistance to parasitoid attack can vary, biological control would benefit from investigating the immune resistance under different field conditions. Ultimately, for immediate within-generation control, parasitoids displaying non-reproductive host killing could be used in pest management by inundative application (i.e., the release of large numbers of natural enemies for achieving a rapid effect), as they can cause substantial mortality in host larvae. For multi-generational effects, they can be supplied by using a banker system, providing them with alternative (non-harmful) hosts to support the population growth of the biocontrol agent (e.g., providing a susceptible host like *D. melanogaster* as a reservoir in the case of *L. heterotoma*).

**Conclusion**

Our results provide an example of how evolutionary principles can be applied to optimize performance of native species for biological control. We consider the evolution of host-range as a stepwise process, and assessed whether intermediate steps can be selected for. This adds to the growing body of evidence that natural enemies can evolve to overcome host defences and be optimized for biocontrol (e.g. Kraaijeveld et al., 2001; Henry et al., 2008; Dennis, Patel, Oliver, & Vorburger, 2017; Lirakis & Magalhães, 2019; Rossbacher & Vorburger, 2020). Even though we did not observe a major effect of artificial selection, wasps that exhibited non-reproductive host killing can influence host population dynamics (Kaser et al., 2018) and thus be an asset for biological control. Host specific factors, such as resistance and condition, will be crucial for the likelihood of adaptation in natural
populations of natural enemies by affecting the reproductive success and thus parasitoid fitness. Empirical and theoretical studies are required linking traits underlying multi-host parasitization and direction of selection pressures in nature to understand and predict the response of native natural enemies to novel invasive species and their eco-evolutionary consequences.

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Chapter 5 | Artificial selection for non-reproductive host killing
Human: “Why do you make such stupid decisions?”

“Do I?” – the wasp