Chapter 4

The role of the endosymbiont Wolbachia and developmental host quality on the parasitization performance of the parasitoid Leptopilina heterotoma attacking Drosophila suzukii

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Abstract

Individual variation among parasitoids in the success rate of host parasitization can arise from both genetic and environmental factors. Here we examined the ability of the parasitoid wasp *Leptopilina heterotoma* to parasitize the non-native pest *Drosophila suzukii*. Previously, we showed that although offspring are generally not able to survive in this resistant host species, wasps are able to reduce survival of *D. suzukii*. Therefore, we are attempting to develop *L. heterotoma* as a biocontrol agent against *D. suzukii* by rearing it on *D. melanogaster*, and then releasing it to induce high mortality in the developing larvae of *D. suzukii*. Heritability analysis indicated that this non-reproductive host killing is not only influenced by genetic variation but also to a large extent by environmental factors. In this study we tested two environmental factors known to affect parasitoids’ fitness: i) developmental host quality (*D. melanogaster*) through manipulation of host nutrition, and ii) heat-shock to remove the endosymbiont *Wolbachia*. Host quality did influence parasitization success: wasps cultured on relatively low-quality hosts produced fewer offspring, these offspring were smaller and exhibited lower killing rate. Three generations of heat-shock treatment did not reduce *Wolbachia* titer and parasitization success, suggesting that this endosymbiont-host association is heat-tolerant. Overall, we demonstrate that phenotypic variation in host-killing rate is affected by host quality, but parasitoids’ offspring survival rate is not improved when attacking *D. suzukii*. This information is important for developing biocontrol programs.

Key-words: endosymbiont, *Drosophila*, host-parasitoid interactions, pest management
1. Introduction

Parasitoids are important natural enemies of insect pests and commonly used as biocontrol agents (Stiling & Cornelissen, 2005; van Lenteren, 2012). The pest is killed through feeding and development of the immature parasitoid inside the host or the consumption of the host by the adult parasitoid (Carton et al., 1986; Jervis & Kidd, 1986; Godfray, 1994). The parasitoids’ ability to control pest populations is determined by a complex set of interacting factors. Among these factors are the genetics of the parasitoid, its associated microbiome, environmental effects, genetics x environmental interactions and various interactions with the phenotype of the pest (Kruitwagen et al., 2018; Brinker, Fontaine, Beukeboom, & Salles, 2019). As a result, understanding and predicting the evolutionary ecology of host-parasitoid interactions and the efficiency of parasitoids for regulating host populations is a major challenge in the field of biological control.

In this study we focus on the parasitoid *Leptopilina heterotoma*, which is a relatively virulent larval parasitoid of a wide range of *Drosophila* species (Schlenke, Morales, Govind, & Clark, 2007; Fleury et al., 2009). It is also one of the few European parasitoids attacking the invasive pest *Drosophila suzukii* (Chabert et al., 2012; Kacsoh & Schlenke, 2012; Miller, Anfora, Buffington, Daane, Dalton, Hoelmer, Rossi Stacconi, et al., 2015; Stacconi et al., 2015; Knoll et al., 2017; Ibouh et al., 2019; Rota-Stabelli et al., 2020; Kruitwagen, Wertheim, & Beukeboom, 2021). When they parasitize *D. suzukii*, however, they seem unable to overcome the relatively high level of immune resistance of *D. suzukii* compared to other *Drosophila* species (Kacsoh & Schlenke, 2012; Poyet et al., 2013; Iacovone et al., 2018). Consequently, parasitization typically does not result in offspring (Kruitwagen et al., 2018; Kruitwagen et al., 2021). Yet, *L. heterotoma* exhibits non-reproductive host killing, defined as parasitoid-induced host mortality without offspring production, indicating that *D. suzukii* is vulnerable to this parasitoid in the invaded area. Moreover, *L. heterotoma* populations vary in host-killing rate (Kruitwagen et al., 2021). This raises the question what hampers this parasitoid species to expand the range of hosts it can successfully exploit for reproduction and what determines individual differences in the magnitude of host-killing rate in *L. heterotoma*. Previously, we reported that host-killing rate has a heritability of 0.15-0.28 (Kruitwagen et al., 2021). Hence, besides presence of significant genetic variation, host killing is also greatly influenced by environmental factors. Here, we address two environmental factors known to play an important role in shaping the parasitoids’ parasitization performance: endosymbiont bacteria and the influence of the host quality for the fitness of the parasitoid that emerges from it.

Natural populations of *L. heterotoma* are infected with *Wolbachia* bacteria (Vavre, Fleury, Lepetit, Fouillet, & Boulétreau, 1999; Vavre, Fleury, Varaldi, Fouillet, & Bouleatreau, 2000), which is a maternally inherited endosymbiont common in arthropods (Werren, Windsor, & Guo, 1995; Werren & Windsor, 2000). *Wolbachia* can have different phenotypic effects on their host, such as reproductive manipulation and provisioning of pathogen protection to facilitate its spread (Zug & Hammerstein, 2015). *Wolbachia* infection imposes large fitness costs in *L. heterotoma*, reducing its fecundity, locomotor activity and adult survival (Fleury, Vavre, Ris, Fouillet, & Bouleatreau, 2000). Moreover,
Fytrou et al. (2006) reported that infected wasps have a significantly lower offspring survival rate in *D. simulans* - a host with a relatively high level of resistance - compared to *Wolbachia*-free females and hypothesized that infection might influence venom production. These results suggest that *Wolbachia* is an important factor influencing parasitization success and may hamper exploitation of *D. suzukii* by the wasps.

External conditions such as temperature can affect the symbiont infection status of hosts (Corbin, Heyworth, Ferrari, & Hurst, 2017). High and low temperatures can reduce *Wolbachia* abundance and alter their impact on their host (Mouton, Henri, Charif, Boulétreau, & Vavre, 2007; Ulrich, Beier, Devine, & Hugo, 2016; Ross, Ritchie, Axford, & Hoffmann, 2019). Symbiont-mediated phenotypic changes might therefore affect the outcome of host-parasitoid interactions with cascading effects on the dynamics of the ecological community that parasitoids are part of. This implies that this temperature-sensitivity feature may be used to manipulate *Wolbachia* presence in potential biocontrol agents. Here, we aim to assess whether reduced *Wolbachia* titers improve the killing efficiency and reproductive potential of *L. heterotoma*. We expect that heat-shock will eliminate *Wolbachia* and that this in return will reduce *Wolbachia*-mediated physiological costs in the parasitoid. Elimination of *Wolbachia* might increase the parasitoids’ ability to exploit *D. suzukii*, by either increasing the rate of non-reproductive host killing or offspring survival.

Another important environmental factor influencing parasitoid fitness is the nutritional quality of the developmental host, i.e., the host in which the female parasitoid developed as larvae. Parasitoids, including *L. heterotoma*, acquire resources mainly as immature stages by feeding from the host tissue, whereas adults often lack the ability to convert and store nutrients in form of lipids for long-term energy storage (Eijs, Ellers, & van Duinen, 1998; Visser & Ellers, 2008; Visser et al., 2018). Indeed, studies show that the state, species or condition of the developmental host has a major impact on the adult phenotype, including nutritional reserves (fat reserves) (Eijs et al. 1998) and adult size (Harvey, Harvey, & Thompson, 1994; Harvey, 2005), of which the latter is an important determinant of fitness and reproductive potential in insects (Vinson & Iwantsch, 1980; Godfray, 1994; Visser, 1994; Kazmer & Luck, 1995; Rivero & West, 2002; Beukeboom, 2018). In parasitoids, body size is reported to influence host searching efficiency, longevity and egg load (Visser, 1994; Kazmer & Luck, 1995; Rivero & West, 2002; Bezemer, Harvey, & Mills, 2005). Likewise, the condition of the developmental host may also influence body size in *L. heterotoma*. This might in turn induce phenotypic variation in killing rate and the ability of offspring to survive on *D. suzukii*.

In this study we investigate whether *Wolbachia* infection and parasitoids’ body size influences host killing rate and offspring survival in the parasitoid *L. heterotoma* exploiting *D. suzukii*. *Wolbachia* presence was manipulated by a heat-shock treatment and wasp body size by rearing wasps on different ‘quality’ hosts through rearing them in a nutritious poor or rich environment. To this end, we subjected parasitoids for three successive generations to heat-shock treatment and next offered these heat-treated parasitoids hosts that differed in quality. The developmental host we used was *D. melanogaster*, which - unlike *D. suzukii* -
is a highly susceptible and suitable host for *L. heterotoma* development. The differentiation in quality was induced by manipulating the nutritional conditions for these hosts. The offspring were then examined for the presence of *Wolbachia*, and parasitization performance was quantified and compared to individuals that were not exposed to a heat-shock. Moreover, we measured parasitoids’ body size to test the hypothesis that low host quality and *Wolbachia* infection would reduce the wasps’ size and to test whether there is a positive relationship between wasp size and parasitization ability of *D. suzukii*.

2. Material and Methods

Insect cultures

Seven European strains of *L. heterotoma* (two from Spain, two from the Netherlands and three from France) were crossed to generate a genetically diverse laboratory culture (this thesis Chapter 3). Wasps were maintained on the relatively low resistant host *D. melanogaster* strain (WW) and reared in plastic bottles containing 30 ml medium (“WW medium”) (agar (17g/L), yeast (26g/L), sugar (54g/L) and nipagine (16.7 ml/L)). For experimental tests, a *D. suzukii* strain was used from Westland, the Netherlands, collected in 2016. *Drosophila suzukii* was reared on “DS” food medium containing 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 (2g/L). Flies were maintained at 20 °C, under a light-dark regime of 16:8.

Heat-shock treatment and manipulation of developmental host quality

To remove the endosymbiont *Wolbachia*, female *L. heterotom* were subjected to a heat-shock during three successive generations (P, F1, F2). First, the laboratory culture was divided in a heat-shock (HS) and a control (HS-C) group, each consisting of 59 randomly chosen females, kept individually. Each generation, 3–10-day old females of the heat-shock treatment (HS) were treated twice with 4 hours in between. The heat-shock was induced by placing individual wasps in 1.5 mL PCR tubes in a PCR machine (Thermo Fisher Scientific, Veriti™) at 37°C for 30 minutes with the cover heating of the PCR machine turned off. Pilot experiments showed that females generally survived this temperature treatment, and in a related system (*Asobara japonica*) a similar temperature treatment (38 °C) reduced *Wolbachia* titer (Brinker P, and Basu M., unpublished results). Females of the heat-shock control group (HS-C) were given the same treatment but at 25 °C. Next, individuals of both HS and HS-C were placed at 25 °C with a male (sibling) for several days and cultured on *D. melanogaster* to produce a new generation. This host species was chosen because the wasps have high (>80%) survival rate on this low resistant host species, whereas on *D. suzukii* offspring generally fail to develop. Wasps were cultured by placing each wasp separately on a batch of ±30 second instar *D. melanogaster* larvae in a vial with *Drosophila* medium (“WW” medium). This allowed tracking of each individual female line over generations. Offspring were allowed to mate with their siblings and then two females were randomly selected per line (one served as back-up to secure the survival of each line)
to undergo the same temperature treatment as their mothers (HS or HS-C); subsequently, they were again cultured on *D. melanogaster*.

After three generations of heat-shock (F3), 20 wasp lines of the HS and 28 lines of the HS-control group were left of the initial 59 lines per heat-shock treatment group. Lines were lost due to mortality before reproduction or lack of female offspring. To test the influence of developmental host conditions on offspring size and parasitization performance, the F3 generation was cultured on hosts that differed in quality using a ‘split brood’ design. Females (2-4 per line) were placed first on the low- or high-quality hosts for 3 hours at 25°C, and two days later on the patch of the other host-quality. Following Fellowes, Kraaijeveld, and Godfray (1998) “low quality hosts” (L) were obtained by placement of 30 second instar *D. melanogaster* on batches with relative low food levels (0.05 ml of 1:4 baker’s yeast and water), whereas “high quality hosts” (H) had access to abundant amount of food (0.5 ml baker’s yeast paste) on a layer of agar. These food levels were shown to induce minor or major larval competition and host mortality rates respectively (Fellowes et al., 1998; Kraaijeveld & Godfray, 1997). This resulted in 4 treatment groups: HS+L, HS+H, HC-C+L, HC-C+H. After culturing on the low- and high-quality hosts, the number of wasp lines decreased to 15 (HS) and 14 (HS-C) (F4). The offspring’s ability to parasitize *D. suzukii* and their body size were measured (see below).

**Parasitization performances measurements**

To compare the effect of developmental host quality and heat-shock on parasitization performances, females of the four treatment groups (F4) were tested for their ability to parasitize *D. suzukii*. Individual parasitization performances were measured following a standardized parasitization performance test as described in this thesis 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. Control vials were maintained on each testing day to measure host survival in absence of the parasitoid. Each wasps’ killing rate was then quantified as the percentage of flies killed in excess to the mortality of non-exposed flies. As measure of reproductive success, successful parasitization was calculated as the proportion of flies killed that yielded wasp offspring.

**Wasp size**

To test the influence of developmental condition on parasitoid body size, and the correlation between wasp size and parasitization performance, we measured tibia length of females of the four treatment groups (F4). Three separate pictures were taken from the right-hind tibia of each wasp with a microscope (5x magnification, Zeiss Stemi SV6) and length (mm) was measured in Adobe Photoshop with the ruler tool. Wasps were always placed on their right-hand side and each photo included a sizing standard by taking a picture of a millimeter paper to set a millimeter scale in Photoshop that sets for each 405 pixels to be 1 millimeter. Average tibia length was used as proxy for body size.
Wolbachia detection

For the detection of the endosymbiont Wolbachia, genomic DNA was extracted from the whole body of each adult wasp, using a high-salt protocol adjusted from Aljanabi and Martinez (1997). To test if the heat treatment was successful in removing Wolbachia infection, presence of Wolbachia was detected by a diagnostic polymerase chain reaction (PCR) using the universal Wolbachia primers wsp-81F (5′-TGG TCCAATAAGTGATGAAGAAAC-3′) and wsp-691R (5′-AAAAATTAAACGCTACTCCA-3′) (Braig, Zhou, Dobson, & O’Neill, 1998; Zhou, Rousset, & O’Neill, 1998), resulting in an amplified DNA fragment of about 600 bp. This test was performed on the 15 surviving lines that had received the heat-shock in three successive generations (F4), as well as in the 15 parental lines (P) from which these F4 individuals originated before the heat shock treatment. PCR amplifications were performed in 20 μL reactions containing 2 μL of DNA (10 ng), 2.5 μL 10x reaction buffer, 2.5 μL dNTPs (2 mM), 1 μL of each primer (10 μM), 0.1 μL of Taq polymerase and 10.9 μL water. PCRs were run under the following cycling conditions: denaturation by 94 °C for 2min, followed by 35 cycles of 60 s at 94 °C, 60 s at 55 °C, 60 s at 72 °C and a final extension step of 10 min at 72 °C.

Statistical analysis

Killing rate was 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, Mächler, Bolker, & Walker, 2014). The effect of heat-treatment and host-quality was analysed by fitting fly survival as dependent variable and both treatments and their interaction as fixed factors and measurement date and female line as random factor. Killing rate was 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. If overdispersion was detected, observation level random effect was added (Harrison, 2015). Parasitoid body size was analysed with linear mixed models in which heat treatment, host-quality and their interaction were fitted as fixed factors and measurement date and female line as random factor. Prior to analysis, the data were Box-Cox transformed to meet assumption of normality (Box & Cox, 1964). The power term of the Box-Cox transformation was estimated with the MASS package in R (Venables & Ripley, 2013), yielding λ= 1.6. Significance of main effects in each model was tested by comparing the full model to the model without the fixed effect by ANOVA. Tukey tests were used for post-hoc comparison of means using the emmeans package (Lenth, 2020). All analysis were done in R (version 4.0.2) (R Core Team, 2020).

3. Results

Quality of developmental host

To manipulate the ‘quality’ of the developmental host, larvae of D. melanogaster were cultured in a poor or rich nutritious environment. Food abundance significantly affected
larva-to-adult survival rates of *D. melanogaster* (GLMM, $\beta = -4.44$, $\chi^2(1)=838.22$, $p<0.01$). On average 88.3% ± 2.34 SE flies survived (out of a batch of 30) when food was abundant, whereas only 17.0% ± 5.12 SE survived when having access to low food level (Fig. 1). Hence, host condition was indeed significantly affected by the food treatment. These hosts of low or high quality were subsequently used to produce a generation of parasitoid wasps that were assessed for (1) *Wolbachia* presence (2) wasp body size and (3) ability to parasitize *D. suzukii*. Prior to this, the parasitoid lines had either received two heat-shock treatments (separated by 4 hours) during three successive generations to remove the endosymbiont *Wolbachia*, or had been reared for three generations without heat-shock as control.

After culturing on the low- and high-quality *D. melanogaster* hosts, the number of wasp lines decreased from 20 to 15 (HS) and 28 to 14 (HS-C) (F4). This was because parasitoids cultured on low quality hosts had a significantly lower survival compared to those from high quality hosts, and the percentage of 30 hosts that gave rise to one parasitoid offspring was 20.3% ± 0.77SE on low quality hosts vs 56.6% ± 1.03SE on high quality hosts (GLMM, $\chi^2(1)=47.54$, $p<0.01$). The number of offspring was neither influenced by the heat-shock treatment (GLMM, $\chi^2(1)=0.13$, $p=0.718$) nor by the interaction between heat-shock and quality of the host (GLMM, $\chi^2(1)=0.014$, $p=0.91$). The proportion of *D. melanogaster* flies killed that yielded a wasp did not differ between food treatments (GLMM, $\chi^2(1)=1.088$, $p=0.30$), heat-shock treatments (GLMM, $\chi^2(1)=0.37$, $p=0.54$) nor their interaction (GLMM, $\chi^2(1)=1.28$, $p=0.26$). Hence, host quality did affect the number of wasp offspring in the F3, but not the parasitization efficiency of the wasps.

**Figure 1 | Survival rate of developmental host (*D. melanogaster*) under different food quantities. Survival rate was calculated as the percentage of larvae that survived to adulthood out of a batch of 30 hosts. Vertical lines represent standard deviation around the mean (black symbol).**

**Wolbachia presence**

To investigate whether heat-shock treatment and developmental host quality influenced *Wolbachia* presence, each wasp line that had received heat-shock treatments during three successive generations and that was reared on the low- and high-quality hosts (F4) was tested with a diagnostic PCR. *Wolbachia* was present in all wasp lines; both in the parental lines and in all the surviving F4 lines. Hence, the heat-shock treatment did not alter *Wolbachia* presence in the lines that survived temperature treatment.
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Figure 2 | Effect of developmental host quality and temperature treatment on (A) parasitoid offspring hind tibia length (mm) and (B) killing rate of D. suzukii (%). Killing rate was calculated as the percentage flies killed out of a batch of 25 hosts, adjusted for average fly mortality of non-exposed flies. Vertical lines represent standard deviation around the mean (darker symbol).

Parasitoid body size

We measured body size of parasitoid offspring that emerged from different quality hosts of each heat-shock treatment group. Tibia length, as a proxy for body size, was significantly affected by the quality of the developmental host: parasitoid offspring that emerged from low quality hosts had significantly smaller tibia length compared to those emerged from high-quality hosts (Fig. 2A) (0.417mm ±0.0068 SE, 0.573mm ±0.0033 SE resp., LMM, $\chi^2(1)=204.95$, $p<0.001$). Heat-shock treatment of the maternal wasps and its interaction with host quality had no significant effect on wasp tibia length (GLMM, $\chi^2(1)=0.008$, $p=0.93$, $\chi^2(1)=0.963$, $p=0.33$ resp.).

Parasitization of D. suzukii

The differently sized offspring that emerged from the low- and high-quality hosts of the heat-shock treatment groups were tested for their ability to parasitize D. suzukii. Reproductive success on D. suzukii was nearly zero: of the 125 tested wasps, only three (2.4%) were able to produce one offspring when exploiting D. suzukii. However, larva-to-adult survival rate of D. suzukii was significantly reduced when exposed to parasitoids (GLMM, $\chi^2(1)=14.357$, $p<0.01$): on average 65% ± 0.36SE of parasitoid-exposed D. suzukii flies emerged whereas 75% ±0.44 SE of flies emerged when not exposed to wasps. Wasps thus exhibited “non-reproductive host killing”.

There was a significant positive correlation between wasp size and host killing rate (GLMM, $\beta = 1.58$, $\chi^2(1)=4.4054$, $p=0.04$): larger wasps had a higher killing rate (Fig 3). Moreover, wasps that emerged from the high-quality hosts killed significantly more flies
compared to wasps that emerged from low quality hosts (Fig. 2B) (36.5% ±1.72 SE, 30.3% ±2.56 SE resp., GLMM, $\chi^2(1)=3.922$, $p=0.04$). Heat-shock treatment of maternal wasps had no effect on killing rate of their offspring (HS-C: 33.9% ±1.81 SE, HS:35.7% ±2.35 SE GLMM, $\chi^2(1)=0.347$, $p=0.56$), and there was no interaction between heat-shock treatment and the quality of the developmental host (GLMM, $\chi^2(1)=2.908$, $p=0.13$).

Although the heat-shock treatment of F1 – F3 females did not affect the killing rate of the F4 offspring from the high-quality hosts, wasps from the low-quality hosts that received a heat-shock in the preceding generations tended to have a higher killing rate compared to those that did not (36.7% ± 6.78, 27.5% ± 2.06 resp.). Statistical interference was however impaired due to low sample size of the low-quality host treatment (n=11).

4. Discussion

In this study we examined the ability of the parasitoid wasp *L. heterotoma* to parasitize the non-native pest *Drosophila suzukii*. Previously we reported that although reproductive success is nearly zero when parasitizing *D. suzukii*, the fruit fly host does suffer from parasitoid attack, which resulted in significant reduced host survival rate (Kruitwagen et al., 2021). We further found that non-reproductive host killing is highly variable among European *L. heterotoma* populations (Kruitwagen et al., 2021), and similar observations have been made for other host-parasitoid systems (Abram et al., 2016). Little is known, however, about which factors determine variation in non-reproductive host killing (Abram et al., 2019). The variation in observed non-reproductive host killing is in part genetically determined, but there is also considerable influence by non-additive genetic effects (Kruitwagen et al., 2021). Therefore, we here investigated the role of two biotic factors known to be relevant for the parasitoid parasitization performance: (1) the nutritional quality of the developmental host and (2) the endosymbiont *Wolbachia*. 

Figure 3 | Predicted probability (marginal effects) of killing rate (%) in relation to *L. heterotoma* size, as measured from tibia length. The grey area represents confidence interval predicted by the generalized linear mixed model.
We found the developmental host to be an important factor for parasitization performance. Specifically, host quality affected the survival rate of immature parasitoids as well as the size and host killing rate of the adult wasp. Moreover, host killing rate was positively correlated with wasp size: larger wasps exhibit larger host killing rate. Similar to previous studies (Chabert et al., 2012; Kacsoh & Schlenke, 2012; Miller, Anfora, Buffington, Daane, Dalton, Hoelmer, Rossi Stacconi, et al., 2015; Stacconi et al., 2015; Knoll et al., 2017; Ibouh et al., 2019; Rota-Stabelli et al., 2020; Kruitwagen et al., 2021), offspring survival in *D. suzukii* was nearly zero and was independent of wasp body size. Secondly, we hypothesized that heat-shock would cure *Wolbachia* infection and release them from induced physiological costs (Fleury et al., 2000; Fytrou, Schofield, Kraaijeveld, & Hubbard, 2006) increasing their ability to exploit *D. suzukii*. In contrast to our expectations, we found that when adult parasitoids infected with *Wolbachia* were exposed to two short periods of heat stress during three successive generations, this had no effect on *Wolbachia* presence nor on their ability to parasitize the pest.

**Developmental host**

Parasitoids developed on hosts in a poor nutritious environment were smaller and exhibited lower host-killing compared to wasps reared on hosts fed *ad libitum*. Indeed, the condition, species or state of the developmental host is often found to induce plastic changes in immature and adult parasitoids (Vinson & Iwantsch, 1980; Godfray, 1994; Li & Mills, 2004; Colinet, Salin, Boivin, & Hance, 2005; Gao et al., 2016). In nutritious poor environments, hosts likely provide less resources for immature wasps to invest in growth and development resulting in smaller sized females. We here show that size also matters for *L. heterotoma* as larger wasps exhibited higher host killing rate of *D. suzukii*. Possibly, larger wasps are better able to handle the hosts during attack and/or have higher venom production. Note that we may not only change the body size by using low quality hosts, but also other physiological factors in the wasps, potentially influencing their parasitization ability. Dietary restriction might for example affect organ development and energy reserves which can consequently lead to different life-history strategies. Yet, reproductive success was nearly zero in *D. suzukii*, irrespective of size, indicating that the benefits yielding from high quality hosts was not sufficient to overcome host defences.

**Thermal sensitivity of Wolbachia infection**

Heat stress has shown to cure *Wolbachia* in a variety of species within the lethal threshold of its host, including spider mites (Van Opijnen & Breeuwer, 1999), filth fly parasitoids (Kyei-Poku, Floate, Benkel, & Goettel, 2003), booklice (Jia, Yang, Yang, & Wang, 2009) and mosquitoes (Wiwatanaratanabutr & Kittayapong, 2009; Ulrich et al., 2016). Such high temperatures can alter *Wolbachia* morphology (Zhukova, Voronin, & Kiseleva, 2008), deterring bacterial replication and density. In contrast, our results suggest that *Wolbachia* is tolerant to short periods of heat at 37°C in *L. heterotoma*. The influence of temperature treatment is influenced by the interaction between the endosymbiont, its host, the remaining microbiome and environmental conditions (Duron et al., 2008; Brinker et al., 2019). Previous studies have shown that *Wolbachia* thermal tolerance depends on *Wolbachia* strain (Ross et al., 2017), the host genotype (Mouton et al., 2007) and temperature regime
(Stevens, 1989). Moreover, heat stress might only have temporal effects on Wolbachia density: bacterial titer may quickly recover within one generation after its density is reduced due to heat (Ross et al., 2020). As such, it is possible that bacterial titer in the F3 was reduced in our experiment, but that Wolbachia was not entirely removed and could therefore be restored in the F4 when they did not receive a heat-shock. It would require a qPCR to assess whether titer was reduced, as this is not possible with a diagnostic PCR. Hence, in our experiment, the exposure time to heat might have reduced the bacterial density, but not have been sufficient to eliminate the bacteria entirely, enabling the bacteria to replicate and recover quickly after the administered heat-shocks. Alternatively, we might have selected on heat-tolerant Wolbachia strains, as bacteria within the same host species can exhibit high genetic diversity (Duplouy, Couchoux, Hanski, & van Nouhuys, 2015; Russell & Cavanaugh, 2017). This diversity of Wolbachia strains also applies to L. heterotoma: three different Wolbachia types have been reported that can co-occur in the wasp (Vavre et al., 1999; Vavre et al., 2001; Mouton, Henri, Bouletreau, & Vavre, 2003).

**Heat-shock treatment x developmental conditions**

Interestingly, heat-shock treatment of maternal generations seems to increase the parasitoids’ host-killing rate when being reared on low quality hosts, but not the high-quality hosts. Several factors could explain this result. Firstly, we might have selected for the fittest parasitoids. We lost a higher number of parasitoid lines after three generations of heat-shock treatment ($\frac{39}{59} = 66\%$) compared to the control lines ($\frac{31}{59} = 52\%$). The surviving lines of parasitoid wasp lines from the heat-shock treatment exhibited lower parasitization performance (i.e., ability to reproduce on D. melanogaster) and higher mortality rates compared to the heat-shock control group. However, after rearing the F3 on high and low-quality hosts, we lost more wasp lines of the heat-shock control group ($\frac{14}{28} = 50\%$) than of the heat-shock treatment ($\frac{5}{20} = 25\%$). Secondly, heat-treatment might have activated stress-regulating factors in the mothers and have transmitted to their offspring, which consequently exhibit higher host killing rates, for example through increasing their activity level. The effect of the heat-treatment might therefore be especially pronounced when reared on low-quality hosts as this is an extra stress factor; wasps reared on low quality hosts likely have limited/less resources available and/or are more stressed. Parasitoids reared on the high-quality hosts might therefore have showed similar parasitization performance independent of heat-shock treatment.

**Conclusions and implications**

Although Wolbachia is harmful to L. heterotoma (Fleury et al., 2000; Fytrou et al., 2006), their reproductive manipulation (cytoplasmatic incompatibility) enables the bacteria to spread through the parasitoid population (Mouton, Henri, Bouletreau, & Vavre, 2005). Moreover, based on the results of the present study, Wolbachia seems tolerant to short periods of heat stress in L. heterotoma, suggesting that their infection is resistant to high temperature. Hence, heat-shock treatment does not seem to be a useful tool to improve biocontrol efficiency of L. heterotoma. From an ecological perspective, heat tolerance
might, next to reproductive manipulation, be a factor contributing to their high infection rate in the field (Vavre et al., 1999; Vavre et al., 2000). Moreover, Wolbachia density can also be influenced through interaction with other microbes present in the host (Goto, Anbutsu, & Fukatsu, 2006) that can also be affected by the thermal environment (Moghadam et al., 2018). Hence, to understand and predict the endosymbiont-parasitoid interaction under ecological relevant climate conditions, further study is needed to investigate the effect of thermal environment on the Wolbachia - L. heterotoma interaction, including the interaction with the remainder of the microbiome (Brinker et al., 2019). Such knowledge of how the thermal environment shapes Wolbachia prevalence could also have important implications for biocontrol, by influencing Wolbachia mediated performance of the biocontrol agent (Floate, Kyei-Poku, & Coghlin, 2006; Ulrich et al., 2016). To understand the role of Wolbachia in parasitization success, an alternative method could be to use antibiotics. It has to be taken into account that usage of antibiotics however results not only in shift of Wolbachia density but of the total bacterial composition (Chaplinska, Gerritsma, Dini-Andreote, Falcao Salles, & Wertheim, 2016; Ourry et al., 2020; Zhang et al., 2020), whereas heat treatment is less aggressive for the host and disturbs fewer other microbes (Brinker P and Basu M., unpublished results).

Our study demonstrates that manipulation of host nutrition provides a useful method to study the influence of (intra-specific variation in) host state on parasitoid fitness, and size-fitness relationships in parasitoid wasps. This is relevant for breeding parasitoids for their release as biocontrol agents. In particular, our results are in agreement with a large body of literature underlining the importance of regulating the condition of the developmental host to secure the quality of parasitoids during (mass) rearing for their usage as biological control agents (e.g. Bigler, 1989; Dicke et al., 1989; López et al., 2009; Parra, 2009; Kishani Farahani, Ashouri, Zibaee, Abroon, & Alford, 2016). Moreover, from an ecological network perspective, such variation in host condition can also have community-wide consequences. This may arise for example via indirect competition mediated by a shared natural enemy (i.e. apparent competition) (Bonsall & Hassell, 1997; Bonsall & Hassell, 2000). The parasitoid fitness effects induced by one host may have cascading effects on the population dynamics of another host species such as D. suzukii, by changing the strength of the host-parasitoid interaction (i.e. magnitude of host killing). This can then in turn influence the parasitoid population size and consequently the interaction of the former host population. Hence, gaining knowledge about what determines individual variation in parasitization will help understanding and predicting the evolutionary ecology of host-parasitoid communities (Bolnick et al., 2011) and their ability to control insect pests.

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