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Does the seasonal phenotype of *Drosophila suzukii* influence cellular immunity and parasitisation?

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Abstract

Controlling the worldwide invasive pest *Drosophila suzukii* remains a challenge. One promising biological method for managing this pest is the use of larval and pupal parasitoids. Unfortunately, most of the larval parasitoids fail to successfully parasitise *D. suzukii* larvae in laboratory experiments due to the high immunity of the pest. So far, only the summer phenotype (summer morph) of *D. suzukii* has been tested for parasitisation. However, the winter phenotype (winter morph) is the dominant form of *D. suzukii* throughout the year in the northern hemisphere. Therefore, this study investigates the immunity during parasitisation for both phenotypes using the larval parasitoid *Asobara japonica* and the pupal parasitoid *Trichopria drosophilae*. It is the first to compare across all life stages the immunity of the winter phenotype to the summer phenotype of not only *D. suzukii* but also *D. melanogaster*. Our results indicate differences in the immunity between the two phenotypes for larvae, pupae, and adults. However, the degree and direction of these differences were inconsistent across the different life stages of *D. suzukii*. The findings have important implications for the integrated pest management (IPM) of *D. suzukii*.

KEYWORDS
biological pest control, haemocytes, immunity, phenoloxidase, spotted wing drosophila, winter morph

1 | INTRODUCTION

Pest management is an ongoing global challenge that requires effective and sustainable solutions (Omkar, 2016). One possible solution is the use of parasitoid wasps, which have been successfully employed in numerous biocontrol systems. A biocontrol method that can provide timely control is augmentative release, where the natural enemy is mass-reared and released periodically (Lenteren, 2003).

To implement a similar system, research has focused on parasitoids to control the cosmopolitan pest *Drosophila suzukii* in an augmentative biological control approach (Häussling et al., 2022; Knoll et al., 2017; Rossi Stacconi et al., 2015; Wang et al., 2016b, 2021). In contrast to *Drosophila melanogaster*, which aims at rotten fruits, the pest *D. suzukii* can cause quality and high yield losses in ripening and ripe thin-skinned fruits (Bolda & Goodhue, 2010; DiGiacomo et al., 2019; Farnsworth et al., 2017; Mazzi et al., 2017). The cosmopolitan pest is endemic to Southeast Asia and spread to Europe in 2008 (Calabria et al., 2012), North America in 2008 (Hauser, 2011), South America in 2013 (Deprá et al., 2014) and Africa in 2013 (Anfora et al., 2020).

Unfortunately, studies in recent years have shown low parasitisation success of parasitoids on *D. suzukii* larvae compared to

D. melanogaster larvae (Chabert et al., 2012; Kacsoh & Schlenke, 2012). In particular, larval parasitoids from the non-native regions, Europe and America, often have lower parasitisation success than those from native regions, such as China and Japan (Chabert et al., 2012; Girod, Borowiec, et al., 2018; Matsuura et al., 2018). For example, *Asobara japonica* from the native region of *D. suzukii* has a success rate of parasitism (SP) of over 90%, but *Asobara tabida* from France has a SP of 0% (Chabert et al., 2012).

One explanation for the high invulnerability of the *D. suzukii* larvae is probably the high haemocyte load of *D. suzukii* larvae compared to *D. melanogaster* larvae (Iacovone et al., 2018; Kacsoh & Schlenke, 2012; Poyet et al., 2013). Circulating haemocytes are blood cells that are responsible for detecting foreign organisms (e.g. parasitoid eggs) and rapidly forming multilayered capsules (encapsulation, lamellocytes), together with the phenoloxidase-mediated melanogenesis, they are part of the innate immune system (Carton et al., 2008). Interestingly, the invasive strains of *D. suzukii* in Europe and America have an even higher haemocyte load than those in the original distribution area in Asia (Poyet et al., 2013), probably due a bottleneck during the invasion event effect (Facon et al., 2011; Lee, 2002). This high load may enable the immune system of *D. suzukii* to resist parasitisation, particularly by parasitoids from regions outside the native range of *D. suzukii* (Kacsoh & Schlenke, 2012). Surprisingly, despite this high haemocyte load, encapsulation is delayed in *D. suzukii* compared to *D. melanogaster* (Iacovone et al., 2018).

The pest *D. suzukii* has two seasonal phenotypes: A summer and a winter phenotype, also known as summer and winter morphs. Adults of the winter phenotype have a longer lifespan at lower temperatures, have larger and darker bodies (Shearer et al., 2016; Wallingford & Loeb, 2016) and enter a temperature-dependent reproductive diapause (Rossi-Stacconi et al., 2016; Toxopeus et al., 2016; Zerulla et al., 2015). Furthermore, comparisons of the two phenotypes showed that the winter phenotype has an overexpression of detoxification genes in response to insecticides (Seong et al., 2022) and the summer morphs have a higher attraction to food odours (Schwanitz et al., 2022). The winter phenotype is adapted to colder conditions and is, therefore, the dominant phenotype at lower temperatures. In the Netherlands, for example, the winter phenotype is dominant from September to June (Panel et al., 2018). Therefore, the winter phenotype is likely to be the dominant phenotype when population control of *D. suzukii* is required for early spring in continental and oceanic climates. Population density control is more effective in early spring because the population is at a bottleneck after the winter (Rossi Stacconi et al., 2018; Rossi-Stacconi et al., 2016; Wiman et al., 2014). Furthermore, *D. melanogaster* also shows these seasonal phenotypes under the influence of the developmental temperature (Ayrinhac et al., 2004; Bouletreau-Merle et al., 1986; Gibert et al., 2000).

Consequently, for successful biocontrol in the given situation, parasitoids should be able to parasitise the winter phenotype. As mentioned above, only a few parasitoid species can overcome the larval immune response of the *Drosophila* larvae and successfully develop (Chabert et al., 2012; Gabarra et al., 2014; Girod, Borowiec, et al., 2018; Iacovone et al., 2018; Kacsoh & Schlenke, 2012; Wang et al., 2020). The most promising larval parasitoids are *Asobara japonica* (Biondi et al., 2020; Wang et al., 2021) *Leptopilina japonica* (Daane et al., 2016; Girod, Rossignaud, et al., 2018) and *Ganaspis brasiliensis* (Fellin et al., 2023; Wang et al., 2020) as well as the pupal parasitoids *Trichopria drosophilae* (Falagiarda & Schmidt, 2020; Hougardy et al., 2022) and *Pachycrepoideus vindemiae* (Knoll et al., 2017; Wang et al., 2016a). Unfortunately, so far, all parasitisation tests for *D. suzukii* have been performed on the summer phenotype. It remains unknown whether the winter phenotype larvae or pupae have the same immune response to parasitisation as the summer phenotype.

We tested the immune system response to parasitism in winter and summer phenotypes of *D. suzukii* and *D. melanogaster* using the larval parasitoid *A. japonica* and the pupal parasitoid *T. drosophilae*, both potential biocontrol agents (Colombari et al., 2020; Falagiarda & Schmidt, 2020; Girod, Lierhmann, et al., 2018; Häussling et al., 2021; Herz et al., 2021; Matsuura et al., 2018; Wang et al., 2020, 2021). Additionally, we determined the phenoloxidase activity of the larval haemolymph of the two phenotypes of *D. suzukii* and *D. melanogaster* and counted all four different types of haemocytes. The haemocyte counts and the phenoloxidase activity provides an overview of the innate immune system of both phenotypes. Furthermore, we measured the phenoloxidase activity of the haemolymph and counted haemocytes from adult females. This should provide a deeper understanding of adult immunity and allow conclusions about the life cycle of haemocytes.

2 | MATERIALS AND METHODS

2.1 | Insects

Drosophila suzukii were caught in the state of Hesse, Germany, in 2016. The parasitoid wasps *Trichopria drosophilae* were provided by the company 'Bioplanet' in Cesena, Italy. *D. suzukii*, *D. melanogaster*, and *T. drosophilae* were reared on *D. melanogaster* and kept under the conditions described in Häussling et al. (2021). *Asobara japonica* were received from the Department of Evolutionary Animal Ecology at the University of Groningen. They were reared on *D. melanogaster* larvae. The parasitoid wasps were fed with a 50% honey–water solution on filter paper, and both sexes were kept together. The parasitoid wasps were, as usual, 4–6 days old when used in the experiment, so they were mostly mated. The summer phenotypes of *D. melanogaster* and *D. suzukii* and the parasitoids were kept in a climate- and light-controlled chamber at 24°C and 70%–80% RH with a 16:8 h day/night rhythm. The winter phenotypes were kept for the hole development in a climate- and light-controlled chamber at 15°C and 70%–80% RH with an 8:16 h day/night rhythm.

2.2 | Parasitisation experiment

In the parasitisation experiment, we tested the parasitisation rate of *A. japonica* on second instar larvae and the parasitisation rate of *T. drosophilae* on pupae of both fly species. Second instar larvae of the two seasonal phenotypes of *D. suzukii* and *D. melanogaster* were used to test larval parasitisation. The larvae were flushed out of a Drosophila vial onto a fine sieve (300 µm aperture), and 740 medium-sized second instar (L2) larvae were collected with a fine paintbrush under a stereomicroscope and placed in Drosophila Ringer solution to free them from residues (per 1000 mL water: 0.33 g $CaCl_2 \cdot 2H_2O$, 13.6 g KCl, 1.21 g Tris base; pH adjusted to 7.2 with 1 N HCl). After the collection (max. 30 min), the larvae were dried on a precision wipe. For each treatment, 30 dried larvae were transferred to a piece of Drosophila diet in a Petri dish. For the parasitisation treatment, three female *A. japonica* wasps were placed on the Petri dish for 6 h at 24°C. We used three wasps to increase the likelihood that the larvae would be parasitised. The piercing was performed as described in the larval immunity experiment in Section 3.3. For each treatment, nine Petri dishes were prepared. After pupation, each pupa was transferred to each well of a 96-well plate. The plate was covered with a gas-permeable sealing membrane (Breath-Easy® sealing membrane, Sigma-Aldrich) and stored at 24°C. After hatching, the number of emerged wasps, flies and unhatched pupae were counted. The degree of infestation (DI) (Boulétreau & Fouillet, 1982; Boulétreau & Wajnberg, 1986) was estimated using the equation:

$$\mathrm{DI} = \frac{(D_C - D_T)}{D_C} \quad (1)$$

where D_C describes the number of emerged flies in the control group and D_T the number of flies that emerged from the treatment groups (parasitisation, pierced). $(D_C - D_T) < 0$, $(D_C - D_T)$ was set=0. We included piercing for the DI to differentiate between wounding reactions and immune reactions.

In the pupal parasitisation experiment, 15 pupae of either the summer or the winter phenotype of *D. suzukii* or *D. melanogaster* were placed on moist filter paper. The pupae were tanned pupae of similar age. Tanning was visually similar between the phenotypes. This filter paper was in a Petri dish (9.5 cm diameter) and offered to one female parasitoid *T. drosophilae*. The temperature of the parasitisation and storage temperature was either the rearing temperature of the summer phenotype (24°C) or the winter phenotype (15°C) (see Section 3.1), but both phenotypes were observed at both temperatures. The oviposition behaviour of the wasp was recorded for 6 h using a video recorder (Lupustec LE 800 4K, Lupus-Electronics GmbH, Landau, Germany), as described in Häussling et al. (2021), and the number of oviposition events were counted. Multiple repetitions were observed simultaneously, each with its own camera. This method was used for *T. drosophilae* parasitism because, unlike in the *A. japonica* parasitism experiment, the pupae are unable to move, allowing the oviposition of each pupa to be observed. We counted an event as oviposition when the wasp pierced a pupa and did not move for at least 30 s. For *D. suzukii* we had sample sizes of 22 Petri dishes for the winter phenotype (9 at 24°C and 13 at 15°C) and 16 Petri dishes for the summer phenotype (8 at 24°C and 8 at 15°C). For *D. melanogaster*, we had sample sizes of 31 Petri dishes for the winter phenotype (22 at 24°C and 9 at 15°C) and 30 Petri dishes for the summer phenotype (25 at 24°C and 5 at 15°C). The numbers are not equal, as in some of the Petri dishes the parasitisation or the observation failed. As a control, no wasps were added to the Petri dishes (sample size of 9 for the winter and 9 for the summer phenotype of *D. suzukii* and 15 for the winter and 13 for the summer phenotype of *D. melanogaster*). All these pupae were then individually transferred to 96-well plates. These plates were sealed with a gas-permeable sealing membrane (Breath-Easy® sealing membrane, Sigma-Aldrich). Once the insect emerged, we identified whether it was a fly or a wasp and recorded its sex. The parasitisation success was measured by the proportion of infested hosts that give rise to an adult parasitoid (Boulétreau & Wajnberg, 1986).

2.3 | Larval immunity experiment

We counted all immune cells in the larval immunity experiment and measured the phenoloxidase activity after the larvae were parasitised. First, the larvae were collected from the rearing vials in the same way as for the parasitisation experiment (Section 3.2). One hundred of the collected larvae were used directly to extract the hemolymph at time point 0 h (no parasitisation). For each parasitisation and control treatment, 200 larvae per replicate were transferred to a Petri dish with a Drosophila diet. More larvae were transferred to compensate for potential losses due to parasitisation and development.

For the parasitisation treatment, 20 female *A. japonica* were added to the Petri dish for 6 h. The control group received no further treatment until hemolymph extraction. For the pierced treatment, 240 larvae were transferred to a Petri dish. More larvae were transferred for the pierced treatment to compensate for the potentially higher mortality due to piercing. Before and during the piercing, the larvae were transferred to an ice-cooled porcelain plate to reduce larval movement for the piercing treatment. Piercing was performed using a flame-sterilised insect needle in the posterior cuticles without harming the internal organs. The piercing treatment was included in the experiment to test the haemocyte induction for a potential effect of piercing during oviposition in the absence of wasp venom, similar to Kacsoh and Schlenke (2012), as the piercing can induce the production of lamellocytes (Markus et al., 2005). Pierced larvae were transferred to a new Petri dish with a Drosophila diet. The petri dishes of all treatments were closed with parafilm tape and placed in the climate chamber at 24°C under long-day (16:8 h day/night rhythm) conditions until haemolymph extraction.

We extracted the haemolymph from 60 larvae from all three treatment groups at 24 and 48 h. Prior to haemolymph extraction, larvae were collected and washed in Drosophila Ringer's solution. They were then dried on a precision wipe and, in groups of 20 larvae,

transferred to a microscope slide. 1 μL of 5 mM MOPS buffer containing protease inhibitor cocktail (Roche cOmplete, CAS 30827997, one tablet solved in 50 mL of 5 mM MOPS buffer) was added per larvae. The buffer compensates for evaporation and inhibits the protease. For the parasitisation treatment, the number of visual encapsulations were counted. The larvae were then cut below the mandibles with dissection scissors and left in the buffer to bleed for 1 min. The buffer containing the haemolymph from the 60 larvae was collected into a 1.5 mL Eppendorf tube and kept on ice. The tubes were frozen in liquid nitrogen and stored at −80°C. Prior to freezing, 5 μL of the haemolymph was mixed with 15 μL N-Phenylthiourea (0.01% in 1× PBS, Thermo Fisher Scientific, CAS 103-85-5) and 2.6 μL Giemsa-Stain (Sigma, CAS 51811-82-6) and loaded onto a haemocytometer (Neubauer improved, NanoEntek, C-Chip DHC-N01) for the haemocyte counting. The haemocytes were counted and discriminated in 16 diagonally arranged grids of the haemocytometer using a phase-contrast microscope (Zeiss, Axio Lab.A1) at 63× magnification. The counted haemocytes were plasmatocytes, podocytes, crystal cells, and lamellocytes. The counting took place directly after the load of the haemocytometer to avoid the loss of crystals in the crystal cells (Kacsoh & Schlenke, 2012).

We used the ratio of phenoloxidase to the total protein content as a measurement for the phenoloxidase. This ratio was used in order to be independent of the haemolymph concentration in each sample due to the different amounts of larval bleeding. The maximum linear rate of colour change (V_{max}) of the substrate L-DOPA converted by PO was used for the phenoloxidase. Total protein was measured using the Pierce™ Rapid Gold Protein Assay Kit (A53225, Thermo-Scientific™). This kit was used in line with the user guide with 5 mM MOPS buffer as blank. Absorbance was measured using a BioTek® Synergy H1 microplate reader.

We measured the phenoloxidase activity using a 10 mM L-DOPA solution and added 34.4 mg of L-DOPA (Sigma, CAS 59927) to 20 mL 5 mM MOPS buffer. This solution was vortexed for 15 min, followed by 3 min in an ultrasonic tub. To measure the samples, we used a microplate reader and kept the 96-well plate on ice. Samples were defrosted on ice, vortexed and centrifuged briefly on a benchtop centrifuge. 10 μL of 5 mM MOPS buffer was added to each well. This step was followed by adding 10 μL of sample haemolymph or, as a control, adding 10 μL of deionised water. The last 180 μL of ultrafiltered 10 mM L-DOPA solution was added and mixed. The set temperature of the microplate reader was 30°C. The samples were mixed orbitally, and the absorbance at 490 nm was measured every minute for 75 min.

2.4 | Adult immunity assay

For the adult immunity assay, we counted the haemocytes and measured the phenoloxidase for both phenotypes. For this purpose, the haemolymph of the female adult *D. suzukii* or *D. melanogaster* were collected by piercing the thorax of the adult flies. The wings were also removed with forceps. 15 female flies were then transferred to a 0.5 mL Eppendorf tube with a fine cut from a razor blade at the bottom. This tube was then inserted in a 2 mL Eppendorf tube containing 5 μL of MOPS buffer with protease inhibitor so that the haemolymph could drip down into the 1.5 mL tube during centrifugation and immediately mix with the protease inhibitor. The tubes were centrifuged at 4025 *g* rpm for 8 min at 4°C. The haemolymph sample from five tubes was pooled so that one sample contained the haemolymph of 75 female flies. 5 μL was used directly for counting circulating haemocytes. The remainder was frozen in liquid nitrogen and stored at −80°C until the phenoloxidase activity, and the total protein was measured as described in Section 3.3.

2.5 | Statistical analysis

The effect of the seasonal phenotype of *D. suzukii* and *D. melanogaster* on the phenoloxidase activity and the haemocyte counts were analysed using a binomial generalised linear mixed model (GLMMs) using the R package 'lme4' (Bates et al., 2015).

As a random factor for each GLMM, we used the repetition equivalent to one Petri dish. We tested the models for overdispersion. If there was overdispersion, we used a negative binomial model. The degree of infestation of the flies' larvae and pupae, the encapsulation rate and the haemocyte counts and female phenoloxidase activity were compared between the two seasonal phenotypes using the Wilcoxon rank-sum test.

All data were analysed in R 4.1.3 (R Development Core Team, 2008).

3 | RESULTS

3.1 | Larvae

3.1.1 | Degree of infestation of *Asobara japonica*

We found no significant difference in the degree of infestation between the winter and summer phenotypes of *D. melanogaster* and *D. suzukii*, neither in larvae parasitised by *Asobara japonica* nor in pierced larvae (Figure 1 and Figure S1).

3.1.2 | Encapsulation rate

The encapsulation rate gives the percentage of *D. suzukii* or *D. melanogaster* larvae that have a visible encapsulation of an *A. japonica* egg (Figure 2). After 24 h of parasitisation, we found no significant difference in the encapsulation rate between the two seasonal phenotypes of *D. melanogaster* larvae. After 48 h, we found that the winter phenotype larvae had a significantly higher encapsulation rate than the summer phenotype larvae. For *D. suzukii* larvae, we found no significant difference in the encapsulation rate at either time point.

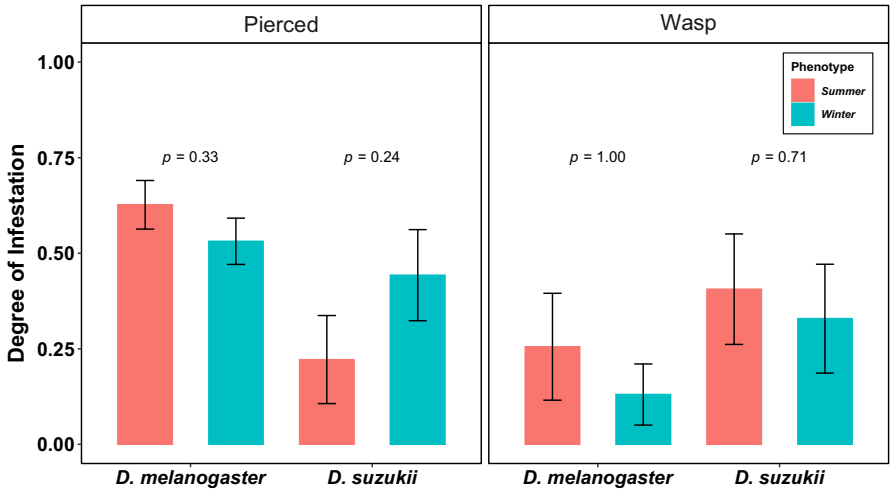

FIGURE 1 Degree of infestation (DI)—the proportion of larvae that could not develop into adult flies due to treatment (pierced or parasitisation treatment) compared with a control group—for larvae of summer (red) and the winter (blue) phenotype of *Drosophila melanogaster* and *Drosophila suzukii* that were either pierced with a needle (pierced) or parasitised by *Asobara japonica* (parasitised) (Wilcoxon rank-sum test). [Colour figure can be viewed at wileyonlinelibrary.com]

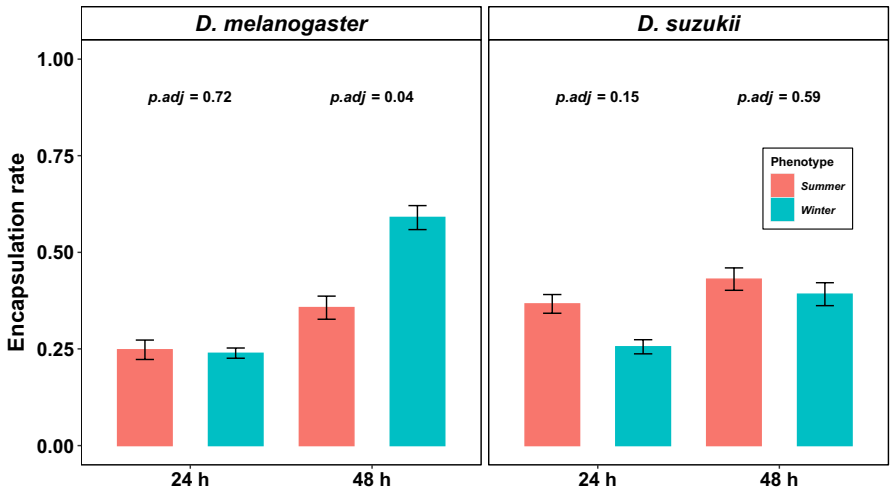

FIGURE 2 Encapsulation rate—the proportion of larvae with visible encapsulations—for larvae of the winter and the summer phenotype of *Drosophila melanogaster* and *Drosophila suzukii*. Encapsulations were counted 24 and 48 h after possible parasitisation. The seasonal phenotype of the larvae was either summer (red) or winter (blue) (Wilcoxon rank-sum test). [Colour figure can be viewed at wileyonlinelibrary.com]

Subset	Response	Terms	χ^2	*df*	*p*-Value
0 h					
D. suzukii	Haemocytes	Phenotype	5.00	1	**0.03**
D. melanogaster	Haemocytes	Phenotype	0.57	1	0.45
24 h					
D. suzukii	Haemocytes	Treatment	5.21	2	0.07
		Phenotype	1.26	1	0.26
D. melanogaster	Haemocytes	Treatment	0.01	2	0.99
		Phenotype	1.41	1	0.24
48 h					
D. suzukii	Haemocytes	Treatment	11.25	2	**0.004**
		Phenotype	5.49	1	**0.02**
D. melanogaster	Haemocytes	Treatment	20.95	2	**<0.001**
		Phenotype	1.05	1	0.31

TABLE 1 Circulating haemocyte counts—generalised linear mixed effect model (family = negative binomial, link = logit, Petri dish ('repetition') as a random factor) output quantifying the effect of the phenotype (winter, summer) and the treatment (control, pierced, parasitisation by *Asobara japonica*) for *Drosophila suzukii* and *Drosophila melanogaster*.

Note: Significant values (*p*-value <0.05) are highlighted in bold. The response 'haemocytes' refers to the total number of haemocytes, with no differentiation of the haemocyte type.

3.1.3 | Haemocyte response of *Drosophila suzukii* and *Drosophila melanogaster* to parasitisation of *Asobara japonica*

In the second instar of *D. suzukii*, we found significant differences between the seasonal phenotypes in the number of haemocytes. Before treatment assignment (0 h), more haemocytes were counted in the summer phenotype than in the winter phenotype. After 24 h, neither the phenotype nor the treatment affected the number of haemocytes. After 48 h, the seasonal phenotype affected the number of haemocytes in *D. suzukii*, with a higher number of cells found in the summer phenotype. At that time, the treatment also influenced the number of haemocytes in *D. suzukii* larvae (Table 1 and Figure 3). Here the 'parasitised' treatment group had a significantly higher number of haemocytes than the 'pierced' treatment group. All other treatment groups were not significantly different from each other (Table S1 and Table 2).

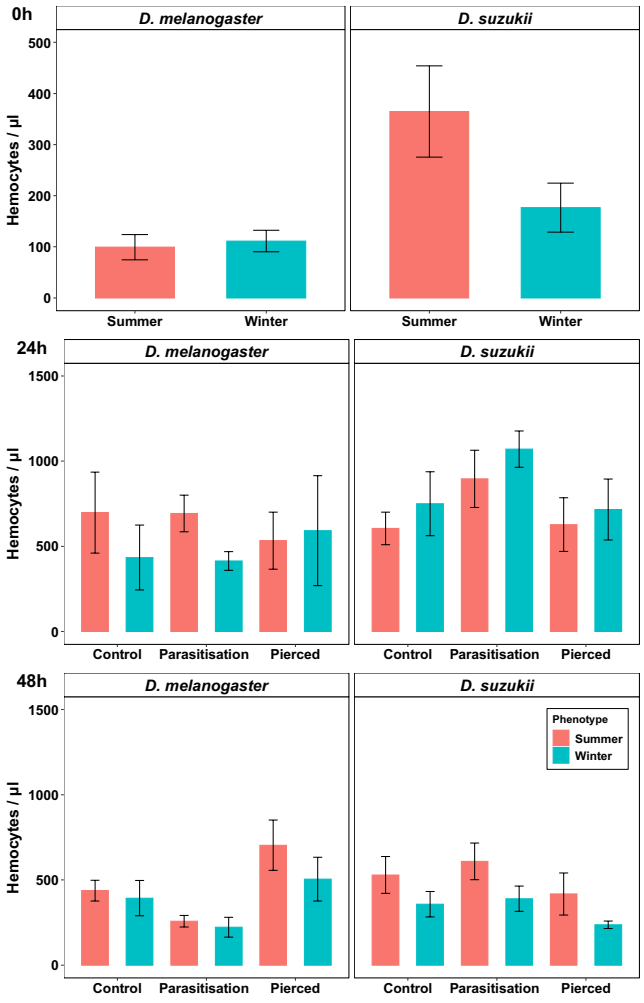

FIGURE 3 Haemocyte count—the number of cells in the haemolymph of *Drosophila melanogaster* or *Drosophila suzukii* larvae. Larvae were pierced or offered for parasitisation or untreated. The seasonal phenotype of the larvae was either summer (red) or winter (blue). [Colour figure can be viewed at wileyonlinelibrary.com]

The haemocyte counts of *D. melanogaster* larvae were not affected by the seasonal phenotype at any time point (Table 1). But the treatment affected the number of haemocytes at 24 h in *D. melanogaster*. The parasitised treatment group had a significantly lower number of haemocytes than the control and the pierced treatment group (Table S2).

Looking at the different types of haemocytes separately, we found that, in *D. suzukii*, the number of podocytes and the crystal cells were significantly affected by the phenotype at 48 h. The summer phenotype had significantly more crystal cells than the winter phenotype (Table S2). In *D. melanogaster*, the phenotype affected the number of podocytes of the second instar larvae (0 h). The winter phenotype larvae had significantly more podocytes than the summer phenotype larvae.

The treatment affected the number of podocytes in *D. suzukii* at the time point 24 h. The parasitisation treatment had significantly more podocytes than the control. At 48 h, the number of plasmatocytes, crystal cells and lamellocytes counts were significantly affected by the treatment (Figures S2–S5). The control group had significantly more plasmatocyte counts than the pierced treatment group. The parasitisation treatment group had significantly more counted crystal cells than the pierced or the control. The same significant differences were observed for the lamellocytes.

For *D. melanogaster* larvae, the treatment significantly affected the number of podocytes and the crystal cell counts at 24 h. More podocytes were found in the parasitisation treatment group than in the control group. In the pierced treatment, significantly more crystal cells were counted than in the parasitisation treatment. At

TABLE 2 Phenoloxidase activity—GLMM (family = binomial, link = logit, 'repetition' (Petri dish) as a random factor) output quantifying the effect of the Drosophila phenotype (winter, summer) of *Drosophila suzukii* and *Drosophila melanogaster* and the treatment (control, pierced, parasitisation by *Asobara japonica*) on the activity of the immune enzyme phenoloxidase.

Time	Terms	χ^2	*df*	*p*-Value
D. suzukii				
0 h	Phenotype	0.55	1	0.46
24 h	Treatment	0.36	2	0.84
	Phenotype	0.69	1	0.41
48 h	Treatment	17.44	2	**<0.001**
	Phenotype	0.78	1	0.38
D. melanogaster				
0 h	Phenotype	2.63	1	0.11
24 h	Treatment	0.41	2	0.82
	Phenotype	1.78	1	0.18
48 h	Treatment	2.28	2	0.32
	Phenotype	0.19	1	0.67

Note: Due to the non-independence of samples from the same Petri dish, we included the ID of the Petri dish as a random factor. Significant values are indicated in bold.

48 h, the treatment significantly affected the number of plasmatocytes, crystal cells, and lamellocytes. The pierced treatment group had significantly more plasmatocytes than the parasitisation treatment group. The pierced treatment had significantly more crystal cells than the control and parasitisation treatment groups. The control group had significantly more crystal cells than the parasitisation treatment group. For the number of lamellocytes, we found that the pierced treatment group had significantly more than the parasitisation or the control groups.

3.1.4 | Phenoloxidase activity

Not affected by the seasonal phenotype at any time point (Table 2, Figure 4, Table S5).

The treatment (control, parasitisation or pierced) also did not affect the phenoloxidase activity in either fly species. The only exception was the phenoloxidase activity of *D. suzukii* at 48 h. Here, the wasp treatment group had significantly lower phenoloxidase activity than the control or pierced treatment groups.

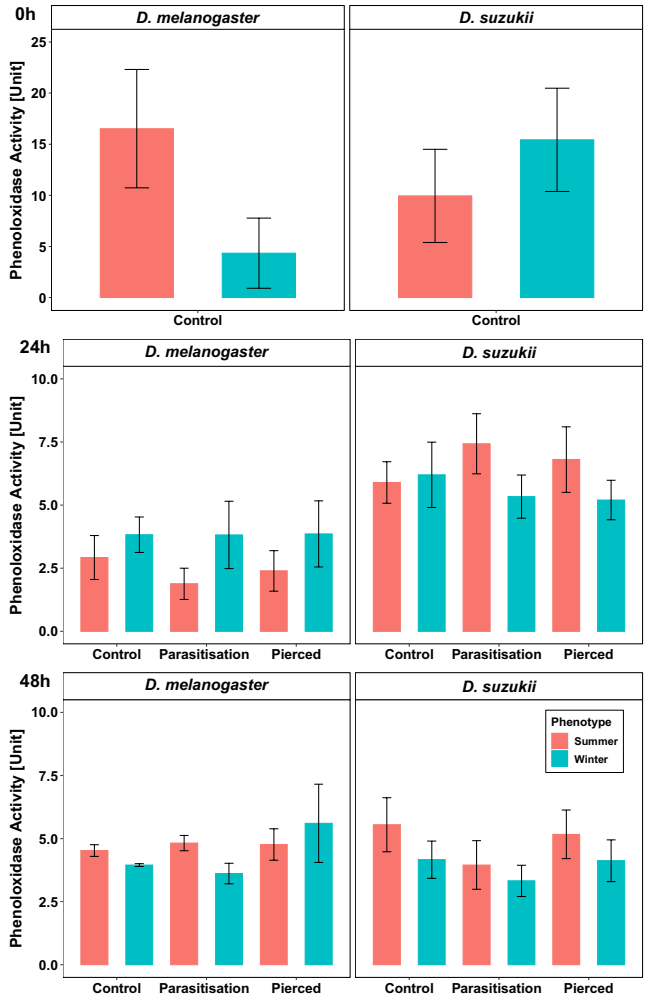

FIGURE 4 Phenoloxidase activity—the ratio of phenoloxidase activity to the total protein in the haemolymph of *Drosophila melanogaster* or *Drosophila suzukii* larvae. Larvae were pierced or offered for parasitisation or untreated. The seasonal phenotype of the larvae was either summer (red) or winter (blue). [Colour figure can be viewed at wileyonlinelibrary.com]

3.2 | Pupae

3.2.1 | Parasitisation success of the pupal parasitoid *Trichopria drosophilae*

Parasitisation success is the number of emerged wasps from previously parasitised pupae. The number of parasitised pupae was for *D. suzukii* at 24°C in summer larvae 98 and 95 in winter; at 15°C in summer, 49 and 46 in winter. For *D. melanogaster*, it was at 24°C in summer, 286, 255 in winter; at 15°C in summer, 11, 16 in winter. The parasitisation success of the pupal parasitoid *Trichopria drosophilae* was significantly different between the two seasonal phenotypes of both fly species *D. melanogaster* and *D. suzukii* pupae at a parasitisation temperature of 24°C (Figure 5). The parasitoid had a higher parasitisation success in the winter phenotype of pupae of *D. suzukii* than in the summer phenotype. In *D. melanogaster*, the summer phenotype was parasitised more successfully than the winter phenotype.

At a parasitisation temperature of 15°C, the parasitisation success of the two seasonal phenotypes was not significantly different for either fly species.

The temperature had no significant effect on the parasitisation success of the *D. suzukii* summer ($p = 0.73$) and winter phenotype ($p = 0.25$). The *D. melanogaster* summer phenotype was significantly more successfully parasitised at 24°C than at 15°C ($p = 0.002$) (Tables S3 and S4). The parasitisation success in *D. melanogaster* winter phenotypes did not differ significantly between those two temperatures ($p = 0.82$).

3.3 | Female adults

3.3.1 | Haemocytes

Adults of the winter phenotype of both female *D. suzukii* and *D. melanogaster* had a significantly higher number of haemocyte counts than female adults of the summer phenotype (Figure 6). In both fly species, the number of haemocytes was much higher in the winter phenotype. The number of haemocytes did not differ between species (winter phenotype $p = 0.72$, summer phenotype $p = 0.59$).

3.3.2 | PO activity

On average, the summer phenotype of *D. melanogaster* had twice the phenoloxidase activity of the winter phenotype. However, the two phenotypes did not statistically differ from each other (Figure 7). In *D. suzukii*, the phenoloxidase activity was equal in both phenotypes. Although not statistically significant, the

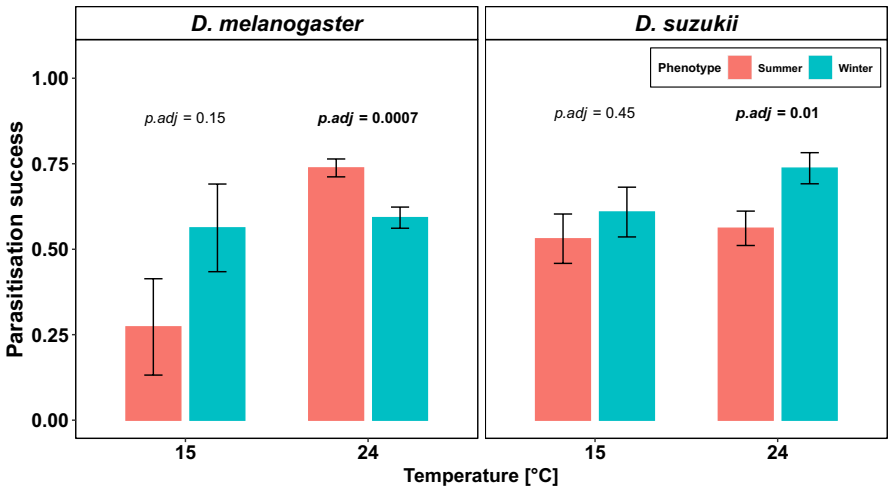

FIGURE 5 Parasitisation success (the proportion of hatched parasitoids) of *Trichopria drosophilae* from pupae of the summer (red) and winter phenotype winter (blue) of *Drosophila melanogaster* and *Drosophila suzukii* at 15 and 24°C (Wilcoxon rank-sum test). [Colour figure can be viewed at wileyonlinelibrary.com]

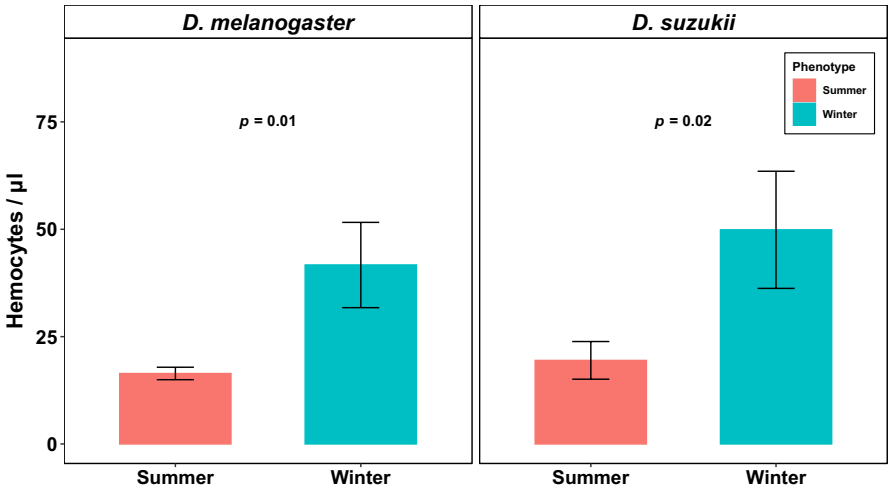

FIGURE 6 Haemocyte count (the number of cells in the haemolymph) of adult females of the summer phenotype (red) and the winter phenotype (blue) of *Drosophila melanogaster* and *Drosophila suzukii* (Wilcoxon rank-sum test). [Colour figure can be viewed at wileyonlinelibrary.com]

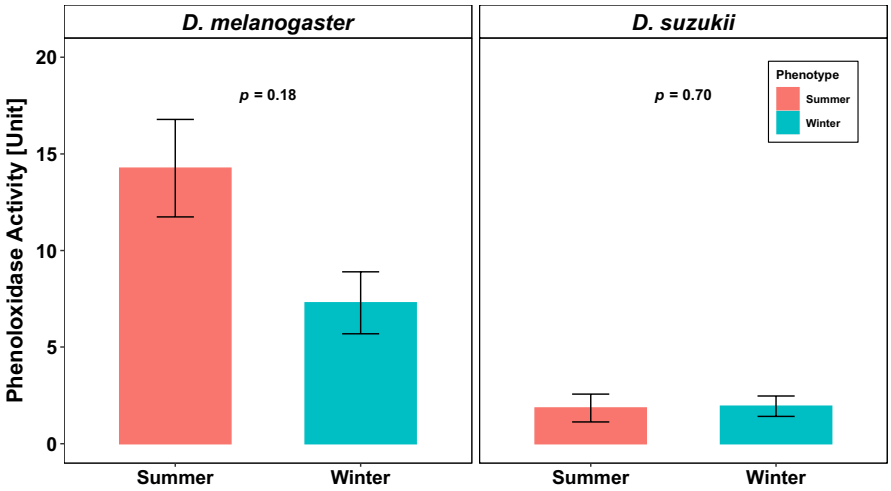

FIGURE 7 Phenoloxidase activity—the ratio of phenoloxidase activity and total protein of the haemolymph of female *Drosophila melanogaster* and female *Drosophila suzukii* adults. The seasonal phenotype of the adults was either summer (red) or winter (blue) (Wilcoxon rank-sum test). [Colour figure can be viewed at wileyonlinelibrary.com]

phenoloxidase activity in the *D. melanogaster* winter phenotypes was almost four times higher than that in the *D. suzukii* winter phenotype. Similarly, for the *D. melanogaster* summer phenotype, the phenoloxidase activity was almost eight times higher than that in *D. suzukii* summer phenotype.

4 | DISCUSSION

In this study, for the first time, the immune system responses to parasitisation of the summer and winter phenotype of different life stages of *D. suzukii* were compared. We found differences

between phenotypes for all life stages, but these differences were inconsistent among the different life stages. Furthermore, we found differences in the immune response between *D. suzukii* and *D. melanogaster*.

First, we looked at the larvae stage. Our results show that at 0 h (second instar larvae), the summer phenotype of *D. suzukii* has a higher haemocyte count than the winter phenotype. This difference could also be found at 48 h after the parasitisation across all treatments (control, pierced, parasitisation). However, the haemocyte counts at 24 h (third instar larvae) after parasitisation do not differ. Thus, at 24 h, when the absolute haemocyte count is highest in *D. suzukii*, the differences between the phenotypes disappear. As we observed the onset of encapsulation at 24 h after parasitisation, the reduced haemocyte count at this time point could be due to a more pronounced encapsulation, because the capsules are an aggregation of haemocytes (Dubovskiy et al., 2016).

Another possible explanation for the reduction in haemocytes could be the temperature: during the parasitisation experiment, we kept both phenotypes at 24°C to keep the wasps active and the results comparable. However, this temperature also meant an increase for the winter phenotype larvae, which were previously kept at 15°C. The effect of temperature on the immune system has only been described for cold temperatures: Salehipour-Shirazi et al. (2017) found an increase in haemocytes in *D. melanogaster* exposed to acute cold. Whether such an increase in temperature, as in our experiment, leads to a similar increase in haemocyte counts should be the subject of further studies.

The immune resistance of *Drosophila* larvae to parasitoid eggs is associated with a high host haemocyte load (Kacsoh & Schlenke, 2012; Poyet et al., 2013). However, our study did not observe a higher immune resistance, as the encapsulation rates and the degree of infestation of the two seasonal phenotypes were similar, also we observed a higher cellular immunity of the summer phenotype. The effect that a high cell count results in higher immunity has also been observed in different strains of *D. suzukii* (Kacsoh & Schlenke, 2012; Poyet et al., 2013). Compared with *D. melanogaster* which has a lower immunity than *D. suzukii*, the haemocyte load in *D. suzukii* strains was five to eight times higher than that in *D. melanogaster* larvae (Poyet et al., 2013). However, in our study, the haemocyte count of the summer phenotype was only twice as high as the winter phenotype. The smaller difference between the phenotypes could explain why the higher haemocyte load did not affect the immunity of the *D. suzukii* summer phenotype in our study.

Furthermore, *Drosophila's* encapsulation process mainly depends on the haemocyte cell type lamellocytes (Binggeli et al., 2014; Dudzic et al., 2015; Vlisidou & Wood, 2015). At 0 h (second instar), lamellocytes were only found in the summer phenotype. At 24 and 48 h post-parasitisation, this cell type did not differ between the seasonal phenotypes, which further explains why we did not find a higher encapsulation rate in the summer phenotype. Another factor that influences the encapsulation is the fly strain, as the encapsulation rate against parasitoids can vary greatly between the *D. suzukii* and the *D. melanogaster* strains (Gerritsma et al., 2013; Kacsoh & Schlenke, 2012). Therefore, it could be that other strains show different encapsulation rates between the two phenotypes.

Drosophila melanogaster phenotypes exhibited no differences in total haemocyte counts or lamellocytes, crucial for encapsulation. Parasitised larvae showed consistent infestation and encapsulation rates across phenotypes. Previous studies noted increased haemocytes, particularly lamellocytes and crystal cells, in response to parasitisation in *D. melanogaster* larvae (Eslin & Prevost, 1998; Kacsoh & Schlenke, 2012). Our experiment confirmed a similar response. In *D. suzukii*, a trend towards higher haemocyte counts in the parasitisation treatment was observed, primarily due to increased lamellocytes and crystal cells after 48 h. This suggests limited impact of wasp venom on *D. suzukii's* immune system. In contrast, *D. melanogaster* exhibited reduced haemocyte counts, especially lamellocytes and crystal cells, indicating the potent effect of wasp venom, consistent with findings by Kacsoh and Schlenke (2012). Overall, haemocyte count differences were more associated with treatment (pierced, control, or parasitisation) than phenotype.

Interestingly, we found encapsulations in *D. melanogaster* and *D. suzukii* as early as 24 h (third instar larvae) after parasitisation. Other studies observed no encapsulation at all when *D. suzukii* larvae were parasitised by *A. japonica* (Iacovone et al., 2018; Poyet et al., 2013) and a delayed encapsulation (compared to *D. melanogaster* larvae) when parasitised by *Leptoplina heterotoma* or *Leptoplina boulardi* (Iacovone et al., 2018). One explanation could be the strains of flies and parasitoids, as we used a different strain of *D. suzukii* and probably also a different strain of *A. japonica* than those studies. As it has been observed that the parasitisation success can vary widely between different parasitoids and *Drosophila* strains from different geographical regions (Gerritsma et al., 2013; Iacovone et al., 2018; Poyet et al., 2013), it is very likely that this factor alone could explain the early encapsulation in our study.

Another important factor in the immunity of *Drosophila* larvae is phenoloxidase (PO), as it contributes to the melanisation process (Dudzic et al., 2015; González-Santoyo & Córdoba-Aguilar, 2012; Moreau et al., 2000; Tang, 2009). We found no difference in PO activity between the seasonal phenotypes in both species. Interestingly, we found a difference between the seasonal phenotypes of *D. suzukii* in the number of crystal cells containing the substrate and enzymes of the phenoloxidase cascade (Carton et al., 2008) at 48 h after parasitisation. However, this difference did not result in higher phenoloxidase activities. Furthermore, the parasitised treatment group of *D. suzukii* had a significantly lower phenoloxidase activity than the control or the pierced treatment groups. This low activity could be due to advanced melanisation of the parasitoid egg in the larvae, where the PO is a key enzyme in melanin biosynthesis (Tang, 2009). In general, there is often no clear correlation between phenoloxidase activity and insect immunity (González-Santoyo & Córdoba-Aguilar, 2012).

We can conclude, that for the larval stage, the difference in haemocyte load in the second instar or 48 h later did not result in any clear immunity benefits, such as a higher encapsulation rate or degree of the infestation when the larvae were parasitised.

In contrast to the larval parasitisation experiment, we found a difference in parasitisation success in the pupae of the *D. suzukii* phenotypes. Parasitisation at 24°C by the pupal parasitoid *T. drosophilae* resulted in a significantly higher parasitisation success in the winter phenotype compared with the summer phenotype. In conclusion, at 24°C, the winter phenotype pupae are more susceptible to parasitism than are the summer phenotype pupae. This finding raises the question of why the two phenotypes have similar degrees of infestation in the larval parasitisation experiment but such different levels of parasitisation success in the pupal stage. One explanation could be the complete morphological change during metamorphosis. Cellular immunity changes at the onset of metamorphosis, resulting in the release of haemocytes by the lymph glands. These cells are part of the metamorphosis when they ingest doomed larval tissues (Holz et al., 2003; Lanot et al., 2001). Therefore, different outcomes between larvae and pupae, as we observed, are possible and, thus, make it difficult to compare these two life stages.

In addition, the pupal parasitoids have evolved different strategies to circumvent the host immune system than larval parasitoids. This difference could cause the observed different levels of parasitisation success of pupa and larval stages. In some species, such as *Asobara tabida*, the egg can stick to host tissue by proteins or special coatings that make the eggs sticky (Eslin & Prevost, 2000; Huang et al., 2021). When attached to host tissue, the egg is less likely to be attacked by haemocytes, which is one passive form of immune evasion. An active form of immune invasion is the venom that is injected during oviposition, which can suppress the host immune response, as shown in *Leptopilina heterotoma* (Huang et al., 2021; Moreau & Asgari, 2015). The virulence of the parasitoid can be species-specific and often also strain-specific (Cavigliasso et al., 2019). To our knowledge, it is unclear whether the parasitoid *T. drosophilae* also injects venom or whether its eggs have a sticky coating. The pupal ectoparasitoid *Pachycrepoideus vindemiae* is known to inject venom into the pupae (Yang et al., 2020). As an endoparasitoid, *T. drosophilae* lays its eggs in the host's hemocoel (Carton et al., 1986). This process means the egg is in contact with the host haemolymph in a similar way to the eggs of larval parasitoids. These behaviours make it likely that the *T. drosophilae* also injects venom, similar to larval parasitoids (Wertheim, 2022).

Temperature affects the immunity of *Drosophila* flies. Cavigliasso et al. (2021) observed a decrease in the encapsulation rate of parasitoid eggs with increasing temperature in *D. melanogaster* larvae. We observed a similar effect in the parasitisation success of the *Drosophila* pupae: With an increasing temperature, a higher parasitisation success of the parasitoid in the summer phenotype of *D. melanogaster* and in the winter phenotype of *D. suzukii* occurred. This effect means these pupae are probably less immune to parasitoids at higher temperatures. In the respective corresponding phenotype, the parasitisation success did not change with increased temperature.

The success of parasitisation relies on both the host's immune response and the parasitoid's ability to parasitise. Temperature, which affects this ability, has been noted in previous studies (Cavigliasso et al., 2021). Further studies evaluating the temperature impact on *T. drosophilae* parasitisation are warranted. Adult females of the winter phenotype in *D. suzukii* and *D. melanogaster* unexpectedly exhibited significantly more haemocytes than their summer counterparts. This contradicts the larval development trend, where *D. suzukii's* winter phenotype had similar or lower haemocyte loads. In *D. melanogaster*, adult haemocytes are primarily embryonic and lymph gland-derived, with little haematopoiesis in adulthood (Boulet et al., 2021; Holz et al., 2003). The observed disparity in haemocyte proportions between adults and larvae suggests potential differences in the phenotypes' ability to maintain haemocytes during metamorphosis, influenced by developmental temperatures. The winter phenotype, developing at 15°C, demonstrated an immunity advantage, as seen in our pupal parasitisation experiment for *D. suzukii*.

Alternatively, the differing decline of haemocytes with age in the two phenotypes may provide an explanation. In *Drosophila*, haemocyte numbers generally decrease with adult age, a phenomenon observed more prominently in females than males (Boulet et al., 2021; Mackenzie et al., 2011; Sanchez Bosch et al., 2019). Surprisingly, adult female flies exhibited lower haemocyte counts than their larval-stage counterparts. This discrepancy suggests a potentially higher decline in haemocytes with age in the summer phenotype compared with the winter phenotype, warranting further analysis.

We found a difference when we examined the phenoloxidase activity. In *D. suzukii*, the phenotypes were not different in PO activity. Additionally, we found a much lower PO activity than in *D. melanogaster*. These findings are unexpected. As *D. suzukii* larvae have a much higher haemocyte load than *D. melanogaster* larvae and a much higher immunity against parasitoids (Kacsoh & Schlenke, 2012; Poyet et al., 2013), we anticipated they would also have a higher PO activity. However, also González-Santoyo and Córdoba-Aguilar (2012) argued in their review that PO activity in insects does not seem to be an indicator of host resistance but rather of host condition, as it is a costly trait. Adult *D. melanogaster* flies live more frequently in food patches with higher population densities than *D. suzukii* (personal observation), and *D. suzukii* avoids competition by shifting the oviposition preference to ripe fruits (Kidera & Takahashi, 2020). In addition, the contact of the fly with microbes should be higher in rotten food patches than in ripe fruits. As a result, the adult *D. suzukii* may have a lower phenoloxidase activity because it is very costly, and the immune challenge is lower in *D. suzukii* adults than in *D. melanogaster* adults.

5 | CONCLUSION

Our study found that differences in immunity between the seasonal phenotypes of *D. suzukii* larvae have no measurable effect on the success of a parasitoid at the larval stage. Still, we suggest further research on the winter phenotype, as other factors than the immunity can affect the parasitisation of *D. suzukii* larvae.

Unexpectedly, we discovered a high haemocyte load in adult females of *D. suzukii* and *D. melanogaster* of the winter seasonal phenotype. This high haemocyte load gives the fly a stronger immunity during overwintering. As far as we know, it is unclear why such a high level of immunity is required for overwintering female flies. At the pupal stage, our study shows that a pupal parasitoid will be more successful on the winter seasonal phenotype of *D. suzukii* when it develops at higher temperatures.

Our results have implications for integrated pest management implementation, where an early release of larval parasitoids during the growing season is crucial to reduce early pest populations. The release of pupal parasitoids may be particularly effective in areas and years where spring temperatures rise rapidly, as the winter phenotype of *D. suzukii* pupae is more susceptible to parasitisation under such conditions. With global warming, these abiotic conditions are expected to become more common in the future.

AUTHOR CONTRIBUTIONS

Benedikt J. M. Häussling: Conceptualization; methodology; software; data curation; investigation; validation; formal analysis; supervision; visualization; project administration; writing – original draft; writing – review and editing. **Nathalie Rausch:** Methodology; investigation. **Emely K. Klüsener:** Investigation; methodology. **Johannes Stökl:** Conceptualization; project administration; supervision; funding acquisition; writing – original draft; writing – review and editing; formal analysis; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Dryad at https://datadryad.org/stash/share/jfWkAQvhoG0MlV0Dowq81SNtHIex9D7sVgHchcTIaCo, reference number doi: 10.5061/dryad.0cfxpnw91.

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