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Overwintering and reproduction biology of *Drosophila suzukii* Matsumura (Diptera: Drosophilidae)

Dissertation

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Contents

The following cumulative PhD thesis comprises the following articles:

[1] Zerulla F. N., Schmidt S., Streitberger M., Zebitz C. P. W., Zelger R. (2015): On the overwintering ability of *Drosophila suzukii* in South Tyrol. Journal of Berry Research **5**(1):41–48.

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[3] Zerulla F. N., Mager N., Zebitz C. P. W. (201x): Effects of different temperatures and photoperiods on diapause induction and status of ovaries of *Drosophila suzukii*. (submitted to Bulletin of Entomological Research).

1. General Introduction

Most fruit flies of the genus *Drosophila* oviposit into damaged, decaying or overripe fruits and vegetables. *Drosophila melanogaster* Meigen is one of the most studied and famous representative in this category (David and Capy 1988). The worldwide distribution of *D. melanogaster* and other Drosophilidae is the result of high fecundity, short generation time, as well as fast adaptability and a wide range of niches and host plants (Ometto et al. 2013). However, the resulting economic loss is negligible, but the spread of a new *Drosophila* pest, *Drosophila suzukii* Matsumura, is of significant concern (Goodhue et al. 2011; Lee et al. 2011). *D. suzukii* is native to east Asia and was detected almost simultaneously in southern Europe and North America in 2008. Since then, it has become a major pest in commercial fruit production areas of the northern hemisphere either by adult migration or after passive dispersal through fruit imports (Cini et al. 2014; Aspen et al. 2015).

Adult morphology

D. suzukii has red eyes, a light brown to yellow thorax and black, continuous stripes on their abdomen (Calabria et al. 2012). Female *D. suzukii* can reach a size of approximately 2.25 to 4.0 mm, whereas males are slightly smaller (2.0 to 3.5 mm) (Kanzawa 1939). The wingspan of an adult female can reach up to 8 mm (Kanzawa 1939; Calabria et al. 2012). The abdominal segments of *D. suzukii* are characterised by large, dark brown to black cross bands that are not interrupted medially (Hauser 2011).

However, two morphological characteristics distinguish *D. suzukii* from other drosophilids (Calabria et al. 2012). Male *D. suzukii* have a characteristic dark spot on each leading wing, which become visible 10 hours up to 2 days after hatching (Fig 1) (Walsh et al. 2011; Anfora et al. 2012) and plays an important role during the mating behaviour (Dekker et al. 2015). Due to these spots, *D. suzukii* is often referred to as "spotted wing drosophila" as English vernacular name (Bolda et al. 2010). Males can also be identified by two short sex combs on the first and second segment of the fore tarsi (Kanzawa 1939; Vlach 2013). The vast majority of *Drosophila* species do not have sex combs; the exceptions include *melanogaster* and *obscura* species (Lakovaara and Saura 1982; Lemeunier et al. 1986; Hu and Toda 2000; Lachaise and Chassagnard 2002; Kopp 2011). Within these species, the sex combs differ in their orientation, the number of teeth and the degree of modification (Kopp and True 2002; Barmina and Kopp 2007). The exact function of the sex combs of *Drosophila* males varies depending on the species (Spieth 1952). However, as part of a stereotypical mating behaviour and tactile interactions between males and females, sex combs are an important component of the mating ritual (Kopp 2011).

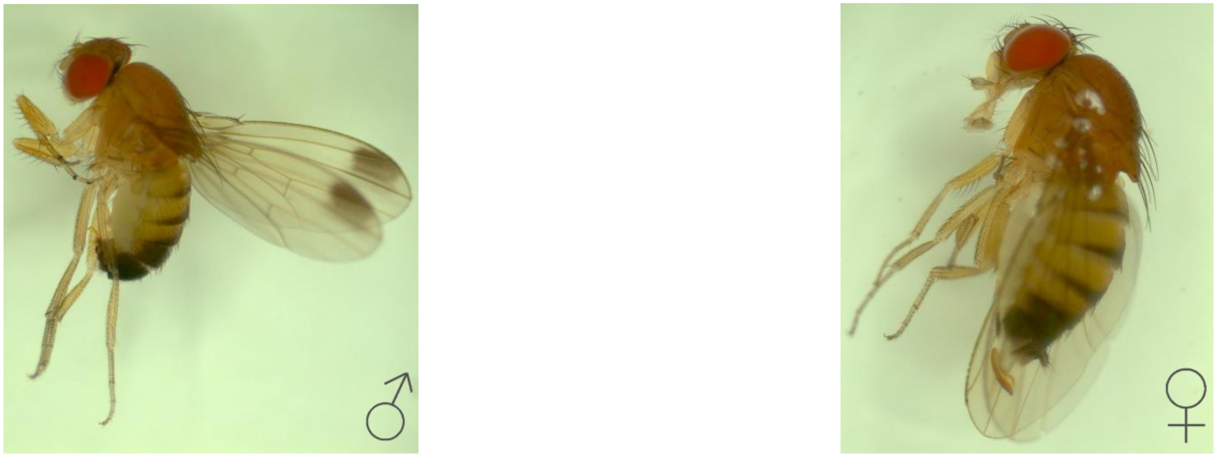

Fig 1. Male (♂) and female (♀) *D. suzukii*

Female *D. suzukii* can be distinguished from other *Drosophila* species by an ovipositor with 60-72 teeth, which differs in size and strength (Fig 2) (Hauser 2011). Comparable high sclerotized ovipositors were found only in some other species of the *suzukii* subgroup, such as *Drosophila subpulchrella* Takamori and Watabe, *D. pulchrella* Tan, Hsu and Sheng and *D. immacularis* Okada (Okada, 1966; Takamori et al. 2006; Atallah et al. 2014). Morphologically, the preimaginal stages of *D. suzukii* are not clearly distinguishable from other *Drosophila* species, but only by DNA-analysis (Hauser 2011).

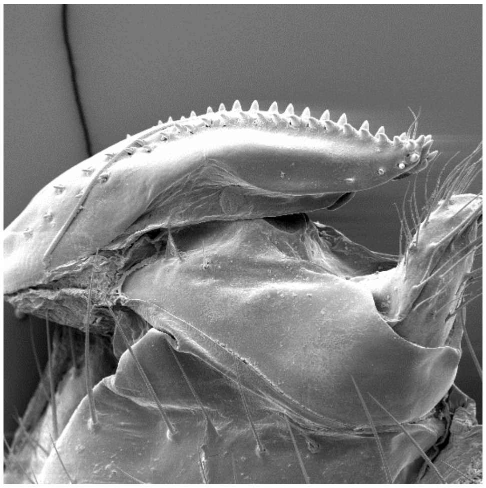

Fig 2. Serrated ovipositor of *D. suzukii*

Fig 3. Egg filaments emerging from an infested blueberry

Previous studies revealed seasonal morphological differences within *D. suzukii* individuals (Stephens et al. 2015; Shearer et al. 2016). This phenotypic plasticity is a phenomenon in which a genotype can lead to several different phenotypes under different environmental conditions (Moczek 2010). It often occurs in insects as a reaction to seasonal changes, in order to react optimally to prevailing climates. This leads to the development of seasonal morphs (Nylin 2013; Shearer et al. 2016). In the case of *D. suzukii*, winter-morph adults have a larger body size and darker pigmentation than summer-morph flies (Stephens et al. 2015). Many organisms grow slower in colder environments, but are larger than adults from warmer conditions (Angilletta and Dunham, 2003). The body size of many ectothermic insects often depends on the ambient temperature. No research has been carried out yet on the environmental factors which are responsible for the phenotypic plasticity of *D. suzukii*. However, temperature has a decisive influence as it is assumed that growth efficiency of individuals decreases with increasing environmental temperature (Angilletta and Dunham 2003; Shearer et al. 2016).

As a further factor to elicit phenotypic changes of individuals dietary proteins have been described. Low protein availability during preimaginal development is often associated with a smaller body size, a slower growth rate and reduced reproductive capacity (Tu and Tatar 2003; Colasurdo et al. 2009; Sentinella et al. 2013; Jaramillo et al. 2014). Due to a faster development time, some larvae can counteract malnutrition by reducing their body size. Thereby the risk of predatory and habitat loss is reduced and the increased mobility as an adult can be used for foraging (Kaspi et al., 2002). In the special case of *D. suzukii*, no reductions in the adult body size have yet been observed due to poor food quality (Jaramillo et al. 2014).

Life cycle

In order to achieve successful reproduction in *Drosophila*, a complex repertoire of male behaviours needs to be executed. For copulation the male has to follow the female, tap her with his front legs, contact the female's genitals with his mouth parts, sing a species-specific courtship song and bend his abdomen (Villella and Hall 2008).

Additionally, pheromones are an important part of the courtship of *Drosophila* species. In contrast to pheromones, which are commonly used for mating among other insects, *Drosophila* pheromones act at short ranges and are not only used for orientation and sexual attraction (Pavlou and Goodwin 2013; Kurtovic et al. 2013). Thus, *Drosophila* pheromones control a variety of sexual and social behaviours (Weng et al. 2013), e.g. increasing the acceptance of males among their sexual partners and reduce the attractiveness of mated females. Virgin *Drosophila* can withstand a male's courtship and be unreceptive by extruding her ovipositor or kicking the wooing male (Pavlou and Goodwin 2013). Moreover, due to pheromones the aggression increases between males willing to mate (Wang and Anderson 2010; Liu et al. 2011). No specific sexual pheromone has been identified for *D. suzukii* yet, but it is known that it does not resemble those of *D. melanogaster* (Dekker et al. 2015). It is assumed that through communication using pheromones and other sensory cues, a female can assess the male's fitness.

However, for successful reproduction, other stimuli, besides pheromones, have to be triggered in the female. The male performs a dance around the female in which he extends his wings and beats them in a high frequency (Pavlou and Goodwin 2013). The vibrations of the flapping wings create a specific courtship song. It is assumed that the spots on the wings of male *D. suzukii* not only cause a visual stimulus but apparently play a decisive role for the courtship song (Bennet-Clark and Ewing 1969; Cook 1979; Hamby 2016).

If a female decides to accept the male she slows down her movements, stops repulsive movements and opens the vaginal plate for mating (Villella and Hall 2008; Pavlou and Goodwin 2013). After the copulation is successful, the mated female becomes temporarily not receptive to further fertilization attempts of other males and increases the oviposition rate (Kubli 2003).

The number of deposited eggs and a successful development to adult *D. suzukii* are strongly dependent on the ambient temperature and environmental conditions (Kinjo et al. 2014). The highest egg deposition of *D. suzukii* was recorded at a temperature of 25 °C, whereas at temperatures below 10 °C and above 30 °C oviposition was completely adjusted (Kinjo et al. 2014; Tochen et al. 2014). A female *D. suzukii* can deposit up to 600 eggs (mean: 380 eggs) and after the adults have hatched, it takes one to three days for the females to deposit the first eggs (Cini et al. 2012; Walsh et al. 2011). However, females are able to oviposit up to 25 eggs per day (Kinjo et al. 2014). The size of *D. suzukii* eggs is about 0.62 mm, they are milky-white coloured and become more transparent during larval development (Walsh et al. 2011).

About one to three days after oviposition the larvae hatch and develop within five to seven days into an adult fly. The hatching larvae is cream-coloured, while their internal organs and black mouth parts are visible due to their transparent shape (Mayr 2012). The mobile larvae undergo three larval instars feeding on fruit flesh of hostplants. After consumption of the fruit where the egg has been deposited, they may switch to another uninfested fruit to continue feeding (Goodhue et al. 2011).

Pupation can take place inside a fruit or on the fruit surface (Bauerfeld et al. 2010). The pupae have a greyish-yellow coloration at the beginning, which changes to a brown coloration when the shell hardens (Walsh et al. 2011; Hauser 2011).

Depending on weather conditions, the total time from egg to adult is about 10 to 79 days, which means that up to 13 generations per year have been found in nature (Kanzawa 1939; Vogt et al. 2012; Tochen et al. 2014). The life expectancy of *D. suzukii* varies strongly, but averages between 20 and 30 days (Tochen et al. 2014), adults older than 60 days were found only rarely in the field (Mayr 2012).

The highest net reproduction rate and population growth was recorded at 22 °C (Tochen et al. 2014). At temperatures between 15 and 20 °C, adult *D. suzukii* show the highest physical activity, while it decreases at temperatures above and below this range (Hamby et al. 2013).

Oogenesis

Insects have different types of oogenesis in different types of variables. In the most primordial form of oogenesis, the panoistic ovaries (e.g. in crickets), yolk is supplied by the epithelial follicle cells and incorporated into the growing oocyte. Thereby, the germ line cysts split into equivalent cells, which become functional oocytes (Pritsch and Buning 1989; Pepling and Spradling 1998, 2001; Kloc et al., 2004). The oogenesis in the panoistic ovary lasts longer (14 days for *Acheta domesticus*) compared to the meroistic variole type (Renucci and Strambi 1983). Meroistic ovaries are characteristic of higher insects and can develop much faster (approx. 3 days in *Drosophila* species), as besides the follicle cells and the oocyte also nutrient cells take over a large part of the synthesis activity (Brubacher and Huebner, 2011; Urbisz and Swiatek, 2013; Tworzydlo et al. 2014).

In general, the oogenesis of *D. suzukii* is not fully investigated. Most of the information is based on research with the closely related *D. melanogaster*. However, in the case of *Drosophila* species it is assumed that the time span varies, until ovary development is completed (Watabe and Beppu 1977). Thereby it is determined by factors such as genotype, age, temperature, humidity, photoperiodism and nutrient availability of adult female flies (Sang and King 1961; Watabe and Beppu 1977). The ovaries are the largest organs in female *Drosophila* species and consist of about

20 clusters of contiguous ovarioles, in which the follicles develop (Gerdeman et al. 2013). Individual follicles develop at the anterior end of an ovariole, the germarium, and go through 14 stages of development as they migrate to the posterior end (King 1970). The first stage is the budding of the egg chamber from germination and stage 14 is the mature egg. This development takes about a week for most *Drosophila* species. All stages of development from stem cells to mature eggs are in a single ovary, whereby each egg chamber contains somatic and germ cells (Bastock and Johnston 2008). The ovaries each end in a lateral oviduct, which unite to form a common oviduct. Located between the ovaries and the uterus, the *receptaculum seminis* (spermatheca) serves fertilisation of the eggs (Fig 5) (Soller et al. 1999). After fertilization, the egg is deposited, and the embryonic development begins immediately.

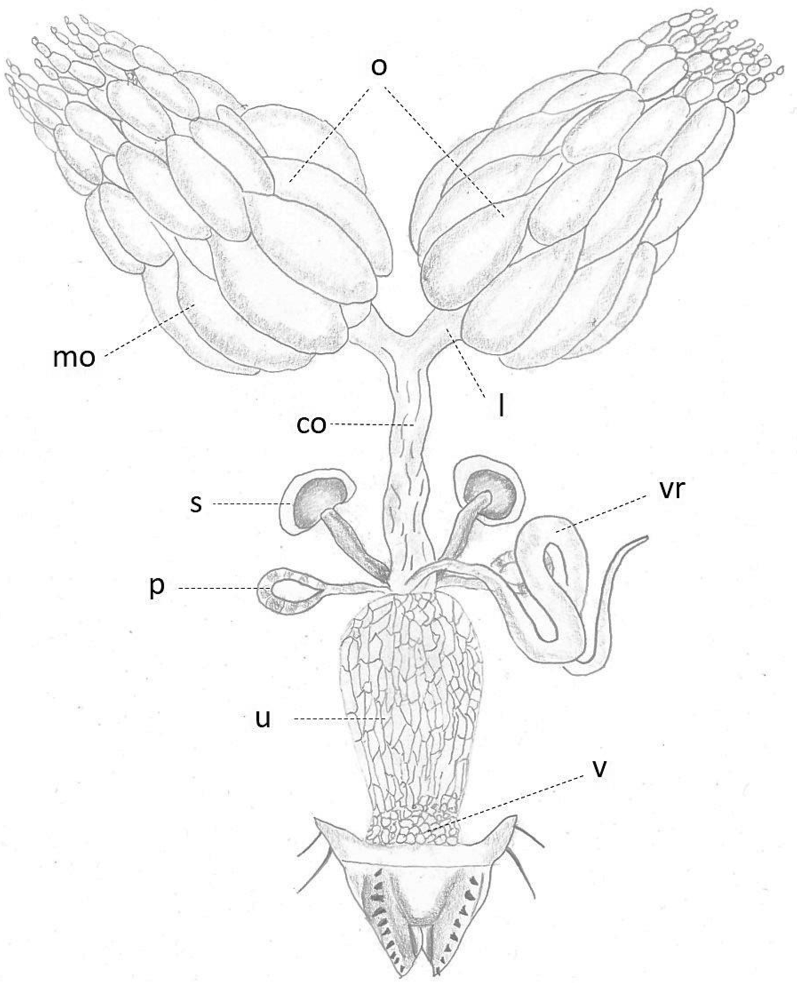

Fig 5. Female *Drosophila* generative organs, ventral view. co, common oviduct; l, lateral oviduct; mo, mature oocyte; o, ovaries; p, parovaria; s, spermathecae; u, uterus; v, vaginal portion of the uterus; vr, ventral receptacle.

Crop damage and pest control

Drosophila suzukii poses a major problem for wine and fruit growers as its serrated ovipositor enables the females to infest soft-skinned fruits, similar to the closely related species *D. subpulchrella*, which is distributed only from southern China to Japan (Fig 2) (Takamori et al. 2006; Atallah et al. 2014). Furthermore, female *D. suzukii* prefer healthy and unwounded fruits with its sclerotized ovipositor (Sasaki and Sato 1995). In addition, ripening fruits are more preferred than overripe ones (Mitsui et al. 2006).

Infested fruits can be detected by two white filaments per egg, emanating from the fruit surface (Fig 3). However, they can easily be concealed by bleeding fruit juice or hair-like structures, similar to those on raspberry surfaces (Little et al. 2016).

The major damage to cultivated crops are a result of wounds due to oviposition, feeding of the larvae and indirectly from secondary pathogens. Other insects (e. g. Vespinae, or the Common earwig *Forficula auricularia* Linnaeus) can benefit from damaged fruit skin caused by oviposition to reach the flesh. Due to larval feeding fruits become very soft and quickly rotten, making infested berries and stone fruits often unsuitable for fresh consumption (Fig 4) (Mazzi et al. 2017).

A very broad host range of wild, ornamental and cultivated plants implies a great population development in the vicinity of vineyards and orchards and also plays a major role in the difficult control of pests (Kenis et al. 2016; Poyet et al. 2015). In addition, a considerable number of host plants such as *Frangula alnus* Mill., *Lonicera spp.*, *Sambucus spp.*, *Vaccinium spp.*, *Vitis spp.*, or *Rubus spp.* were found all over in affected countries (Mitsui et al. 2010; Baroffio et al. 2014; Lee et al. 2015). These hosts are heavily infested every season and of decisive importance of population growth at the beginning of the year (Van Timmeren and Isaacs 2013).

Another key for its successful spread in new regions is the occupation of an empty ecological niche, avoiding major resident competitors and natural enemies (Atallah et al. 2014; Poyet et al. 2015). No beneficial insects were found feeding on *D. suzukii*, so predators are not suited for use as biological control agents. Therefore, a negligible number of parasitized *D. suzukii*-larvae or -pupae were found in field collections across Europe and North America (Burrack et al. 2013). Laboratory studies indicate that *Trichopria drosophilae* Perkins (Diapriidae) and *Pachycrepoideus vindemmiae* Rondani (Hymenoptera: Pteromalidae) are effective pupal parasitoids (Haye et al. 2016). However, due to a heavy immune response against parasitoids, commonly known larval parasitoids of *Drosophila* (e.g. *Amazonides tabida* Guenée, *Leptopilina boulardi* Barbotin, Carton & Kelner-Pillault, *L. heterotoma* Thomson) appear to be unable to parasitize *D. suzukii* (Kacsoh and Schlenke 2012; Poyet et al. 2013). This means, at the present moment, no significant suppression of *D. suzukii* population can be achieved, by using beneficials.

Fig 4. Grapes (Dornfelder) and blackberries infested by *D. suzukii*

Among fast reproduction, high reproduction rates and the availability of host plants, crop loss depend on cultivar, location, microhabitats, vegetation structure and cultivation measures (Van Timmeren and Isaacs 2013; Asplen et al. 2015; Diepenbrock and Burrack 2016; Tochen et al. 2016; Mazzi et al. 2017). Current control strategies of *D. suzukii* mainly base on insecticides which can reduce damage of agricultural crops below the economic injury level, but enhance production costs (Goodhue et al 2011; Cini et al. 2012; De Ros et al. 2013; Mazzi et al 2017). Besides higher production costs, *D. suzukii* also increases economic costs. Depending on the crop, this includes high yield loss and associated loss of sales, as well as labour and material costs to monitor infestation (Goodhue et al 2011). The economic damage varies according to crop, location and intensity of infestation. In California, Oregon and Washington, for example, a total loss of $511.3 million in 2008 was estimated by *D. suzukii* at an average yield loss of 20 % (Goodhue et al 2011). The costs for control management are very difficult to estimate, as labour-intensive measures to control *D. suzukii* are not implemented by every grower in the same way and insecticides are sometimes used to control several insect pests (Bolda et al. 2011; Goodhue et al 2011). At low pest pressure, conventional crop protection strategies could be more profitable than no pest control. If pest pressure increases, however, a combination of crop protection measures like biological, physical, cultural, and chemical control methods are more profitable than single measures (Leach et al. 2016).

Furthermore, only few active, unspecific insecticides are approved for *D. suzukii*-control, such as Cyantraniliprole, lambda-Cyhalothrin, Spinosad and Pyrethrine (BVL 2017), which increases the risk of insecticide resistance. No insecticide specifically active against *D. suzukii* has been developed, yet.

Due to restrictive limitations of application frequency during fruit ripening and harvesting, as well as not to override maximum residue levels, there is a greater chance for the establishment of alternative control strategies (Haviland and Beers 2012; Van Timmeren and Isaacs 2013). Thus, in highly afflicted regions, sales may decline due to the banishment of endangered products from export markets (Mazzi et al. 2017)

Attempts to control *D. suzukii* by pheromones, similar to the control of the grape pests *Lobesia botrana* Denis & Schiffermüller and *Eupoecilia ambiguella* Hübner by mating disruption, have not been successful so far (Louis and Schirra 2001; Landolt et al. 2012). *Drosophila suzukii*'s sexual pheromones are only effective over a very short distance and a combination of visual, auditory and olfactory stimuli is necessary for a successful mating (Bennet-Clark and Ewing 1969; Cook 1979; Hamby 2016). Avoiding infestation of endangered plants by using natural repellent odors, have not been successful (Pham and Ray 2015). However, the main problems are that crop yield is no longer suitable for fresh consumption, or the taste is sustainably and negatively affected. Furthermore, no repellents have been found which could completely stop *D. suzukii* from infesting host plants (Pham and Ray 2015; Renkema et al. 2016).

The most promising alternative to insecticide applications is the netting of plant stocks. In small blueberry and raspberry plantations in North America and for blueberries in Europe, the infestation could be significantly reduced (Kawase et al. 2007; Grassi and Pallaoro 2012; Link 2014; Cormier et al. 2015; Rogers et al. 2016). Thereby, the time of netting is very important. It must be ensured that the crop plants are pollinated and that the pollinators can approach and depart the plant stock (Leach et al. 2016). In case plants are netted too late, *D. suzukii* can already be present in the plant stock and cause serious damage (Rogers et al. 2016). One disadvantage of netting is the high material costs. However, Leach et al. (2016) assume that the net will pay for itself through savings in insecticide treatments over the entire lifespan of netting.

Therefore, an effective long-term control is not possible, but the combination of cultural management tactics such as mass trapping, sanitation or/and by using nets offer a suitable alternative in several agricultural crops (Haye 2016; Mazzi et al. 2017).

Geographical distribution and ecological adaptation

Another reason for the high economic losses is the large number of surviving individuals during the winter in the invaded countries. This causes damage to early ripening varieties in spring, but also to red grape varieties and raspberries in autumn due to a constantly growing population density during the growing season (Stephens et al. 2015, Shearer et al. 2016). In accordance with previous field studies, overwintering is a bottleneck for the survival of *D. suzukii* (Zerulla et al. 2015).

However, insects evolved in different ways avoiding extreme temperatures. They can react by escaping from these extremes and return when conditions become more hospitable (Ramløv 2000). Many insects also hide in microhabitats to avoid the exposure to environmental changes such as extreme temperatures. Insects inhabiting water, soil or forest habitats are then well protected from major changes in ambient air temperature and relative humidity (Johnson et al. 2004; Schowalter 2016)

A reversible change in the morphology of insects, life history, physiology or by adapting to new environmental conditions can be another way to survive in hostile environmental conditions (Schmidt and Conde 2006). For instance, adaptation to low temperatures can take place through a number of morphological, anatomical, biochemical and physiological features.

Adaptation of an invasive pest to seasonal changes in the invaded countries includes an early prediction of the upcoming winter by successive changes of environmental conditions to prepare for the cold season of the year (Tyukmaeva et al. 2011). This plays an important role especially in the early stages of population divergence and for a successful hibernation (Via 2009). Particularly drosophilids have developed various physiological characteristics to adapt to new climates, such as winter robustness and hardiness, diapause and photoperiodism (controlling seasonal life cycles or diapause) (Kimura 1990). Most *Drosophila* species hibernate as adults in different habitats, but also as larvae or pupae in some sort of dormancy (Hoffman et al. 2003; Strachan et al. 2011):

1. Quiescence. The reaction of individual insects to an unexpected, non-cyclical and short-term change in environmental conditions. It is assumed that it is limited to winter-active insects only and results in a growth retardation (Koštál et al. 2006).
2. Oligopause. Describes a fixed resting period in winters of temperate latitudes. This kind of dormancy causes major growth retardation and growth stoppages. The insects need food reserves or periodic winter feeding (Saunders et al. 1989).
3. Diapause. The most advanced resting period for long-term, cyclical weather extremes for surviving seasonally adverse conditions. In most cases, diapause is limited to a single stage of development, but can occur in different species at different stages (Denlinger 2002). The main difference to the other resting stages is also that usually a temperature-independent factor initiates metabolic changes (Giebultowicz and Denlinger 1986; Giebultowicz 2000; Denlinger 2002). Photoreceptors enable insects to differentiate between day and night and, when a threshold value is exceeded, trigger the expression of genes that produce proteins that induce the diapause (Hardie 2001). Therefore, winter is expected long before the onset of cold temperatures, which allows insects to store additional energy reserves because hibernating insects do not feed during winter. The feeding periods correspond to the most favourable time of year, where oviposition takes place (Denlinger 2002).

Drosophila suzukii overwinter as adult winter morphs in forests, entering a reproductive diapause (Kanzawa 1936; Zerulla et al. 2015). This type of diapause allows an organism to save resources while temporarily interrupt reproduction to overcome unfavourable environmental conditions, continuing reproduction when conditions becoming more favourable (Nylin 2013). The development of oogenesis, vitellogenesis, glandular activities and mating behaviour is stopped during the reproductive diapause of adult insects (Tatar and Yin 2001). Additionally, Drosophilids have several other strategies to survive life-threatening cold temperatures and suboptimal humidity, including accumulation of cryoprotectants in their haemolymph, increased expressions of stress induced genes and an increased melanisation (Burton et al. 1988; Ohtsu et al 1998; Trullas et al. 2007; Koštál et al. 2011; Vesala et al. 2012a). It has also been observed that ectotherms, including several *Drosophila* species have a larger body size under cold environments. This phenomenon may play a role for thermoregulation, but this behavioural trait is not yet well understood (Atkinson and Sibly 1997; Karan et al. 1998; Angilletta and Dunham 2003).

As a result of climate change and an increase in average global temperature the damage caused by invasive pests will probably intensify in future (Shearer et al. 2016). A higher annual average temperature lead to sharply higher overwintering survival, higher reproductive rates and, during the year, an increasing number of generations (Porter et al. 1991). In the case of *D. suzukii*, knowledge of the factors for a successful hibernation and adaptation under new environmental conditions plays an important role for assessments of infestation pressure and economic losses.

Thesis Objectives

Since *D. suzukii* has become increasingly important as an invasive pest in Europe and North America in 2008, there is limited research on the biology of the pest. In particular, no fundamental research has so far been carried out on hibernation and diapause biology of *D. suzukii*. This involves not only the induction and refraction of the diapause, but especially the postulated reproductive diapause. This appears to be necessary in order to increase the chances of survival of hibernating adults until the oviposition on available fruits in spring ensuring the survival of the population. Although other insects of the subfamily Drosophilidae (reproductive) diapauses have been found (Goto et al. 1998), however, since there is a very large genetic inter- and intraspecific variation in the genus *Drosophila*, no well-founded conclusions can be drawn on the physiological behaviour of *D. suzukii* (Williams and Sokolowski 1993). Within the genus *Drosophila* there are different types of diapause, different diapause-inducing factors and sensitive stages of development as well as different stages of diapause (egg stage, larval stage, pupal stage or as adults) (Salminen et al. 2012; Vesala et al. 2012a). Therefore, the studies of this thesis investigated the overwintering and reproduction biology of *D. suzukii*.

Three main topics were examined in this thesis:

1) The hibernation capability and the overwintering behaviour of *D. suzukii* at different hibernation sites in South Tyrol and the ovarian development of females, which were caught and dissected over the course of the year from 2012 to 2014.

2) The effects of temperature and temperature-conditioning on oviposition activity and behavioural temperature preferences to contribute to a better understanding of the behaviour and complements the effect of temperature on lifetable parameters of *D. suzukii*.

3) The effects of temperature, photoperiod and temperature-photoperiod-conditioning on the reproductive diapause of *D. suzukii*. This research aims to improve knowledge in the adaptation of *D. suzukii* to changing environmental conditions.

2. On the overwintering ability of *Drosophila suzukii* in South Tyrol

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Abstract

Drosophila suzukii became one of the most dangerous pests in fruit crops and vineyards in Europe since 2009. Lacking knowledge of the overwintering biology under European environmental conditions makes an environmentally friendly control of this invasive pest difficult. The objective of this study was to investigate its hibernation capability under the climatic conditions of South Tyrol, Italy. Monitoring flight activity by baited traps revealed an overwintering location in the Adige valley. We determined the reproductive status of females trapped weekly at "Schlossleiten" from August 2012 until September 2013 by dissecting their abdomens. For further potential correlation between overwintering locations, reproductive status, and climatic conditions were assessed. The microclimatic conditions of single locations affected overwintering success and suggest the aggregation of adults in most favourable hibernation sites where a high proportion of females is able to survive. However, in spring some of the females showed degenerated reproductive organs. Dissection of abdomens evinced a reproductive diapause, which appears to be affected by winter climatic conditions, the nutritional status, and food availability. *D. suzukii* is able to overwinter in the South Tyrolean area. Control and management of the potential hibernation sites may delay the population dynamics in the following season.

Keywords *Drosophila suzukii*, overwintering, reproductive status, microclimatic data, flight activity

3. Oviposition activity of *Drosophila suzukii* as mediated by ambient and fruit temperature

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Abstract

The invasive pest *Drosophila suzukii* was introduced to southern Europe in 2008 and spread throughout Central Europe in the following years. Precise reliable data on the temperature-dependent behaviour of *D. suzukii* are scarce but will help forecasting and cultivation techniques. Depending on physico-chemical properties, surface temperature of objects may differ from ambient temperatures, determining physical activity, and affect oviposition on or into substrate, determining preimaginal development later. Therefore, the preferred ambient temperatures of *D. suzukii* and fruit temperature for oviposition were examined on a linear temperature gradient device.

Thirty adults (15 ♀; 15 ♂) were adapted to different temperatures (10, 20, 30 °C) for six days and then exposed to different temperature gradients (10 – 25, 20 – 35, 25 – 40 °C). *D. suzukii* adapted to 10 °C remained in cooler regions and suffered from a significantly higher mortality at the 25 – 40 °C gradient. Animals adapted to warmer temperatures had a wider temperature preference on the gradient device. Acclimation to lower temperatures and the resulting lower temperature preferences may allow the flies to disperse better in spring to search for oviposition sites. The oviposition activity decreased continuously at a fruit temperature above 28 °C and below 15 °C, with highest oviposition activity in fruits with temperatures between 19.7 °C and 24.8 °C. The preferred fruit temperature is in accordance with the temperature optimum of reproduction biology and preimaginal development of *D. suzukii* reported in the literature.

Keywords Spotted Wing Drosophila, fruit temperature, oviposition preference, temperature adaptability, temperature gradient

4. Effects of different temperatures and photoperiods on *Drosophila suzukii* adults’ reproductive diapause induction and termination

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Abstract

Lacking a diapause at the immobile egg or pupal stage, *Drosophila suzukii* hibernates in a reproductive diapause. This work describes the influence of temperature and daylength on reproductive diapause of a shortday- and a longday-adapted *D. suzukii*-population. Mortality, oviposition and ovarian development were assessed for ten days old females moved from regular breeding environments to longday conditions at 10 and 20 °C, or to shortday conditions at 10, 15, and 20 °C for 14 days. Additionally, the populations were kept under shortday and 10 °C conditions for 14 days and moved to longday and 20 °C conditions. Longday-adapted females suffered from a higher mortality than shortday-adapted ones. The highest egg deposition was found when longday- and shortday-adapted populations were kept at 20 °C. Low temperatures appeared to abrogate adaptation to daylength and mean numbers of eggs/female were low and did not differ significantly between shortday- and longday-populations. Adults kept at shortday conditions brought to longday and 20 °C after 14 days for additional 7 days, within three days re-started from no oviposition to similar numbers of deposited eggs as at longday and 20 °C condition. The proportion of “mature eggs” increased with temperature, whereas the proportion of “immature ovaries” was highest when females were kept at low temperatures. The studies have shown that temperature seems to be of particular importance for oviposition and day length determines the development of the ovaries. Furthermore, we suggest that between 10 °C and 15 °C *D. suzukii* enters a phase where oviposition stops.

Keywords Spotted wing drosophila, reproduction, invasive species, conditioning, photoperiod

Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also known as the spotted wing drosophila is an invasive species in Europe and North America from Eastern Asia. Its unique anatomy and biology different from other *Drosophila* species enabled *D. suzukii* to become a serious economic pest in Europe and North America (Hauser 2011; Calabria et al. 2012; Cini et al. 2012, 2014; Asplen et al, 2015).

Its serrated ovipositor allows *D. suzukii* to lay eggs in unwounded, ripening soft fruits and grapes, thereby massively threatening fruit production such as blueberries, strawberries, cherries, plums, peaches, nectarines and grapes (Walsh et al. 2011). Due to a high reproduction rate, a short generation time, tolerance of a wide range of different habitats and lacking natural enemies, *D. suzukii* spreads rapidly and economic losses are severe (Mitsui et al. 2006; Goodhue et al. 2011; Lee et al. 2011; Cini et al. 2012).

Particularly in Europe, where very heterogeneous small scale geographical and landscape patterns characterised by a high variability of (micro)climates prevail, knowledge of temperature adaptation and diapause induction would help to monitor and control *D. suzukii*.

For many invasive insects like *D. suzukii*, overwintering in new environments is a bottleneck in population survival. Insects that live in temperate or arctic regions must be able to survive winter season with long periods of adverse conditions such as extreme cold and lack of food. Diapause, cold-hardiness, long-distance, intercontinental migration to habitats, providing suitable environmental conditions, or entry into a reproductive diapause at a protected hibernaculum are adaptations to ensure survival during seasonally adverse conditions (Andreadis et al. 2005). Diapause induction and termination as triggered by environmental factors maximize synchronization of development and reproduction with seasonally suitable conditions (Ruberson et al. 1998).

Photo-optical cues, such as daylength, light intensity or wavelength, and temperature are used by arthropods to program diapause induction (Denlinger 2002). Diapause termination is mostly triggered by cold temperatures, often in combination with other factors.

Usually, the diapausing stage is fixed within any species, commonly represented by immobile stages as egg and/or pupal diapause. In male and female adults, reproductive diapause describes halted reproduction in mature insects, where the individuals are physically quiescent and metabolism is reduced to save energy to survive. Reproduction recommences when the reproductive organs have recovered and environmental conditions allow the offspring to develop successfully (Lees 1955; Baker and Russell 2009).

Understanding the overwintering-biology of insects allows the prediction of their behaviour and how they might adapt to a new environment. Particularly in invasive species, the detailed understanding of their ecology helps to predict the probability of their establishment in the new environment, to develop control strategies or to forecast seasonal population dynamics based on bioclimatic models (Cini et al. 2012; Asplen et al. 2015).

As a perfect adaptation to temperate climates, characterized by warm summers and cold winters *D. suzukii* does not survive winter in a diapause at the immobile egg or pupal stage(Rossi-Stacconi et al. 2016). *D. suzukii* adults stay quiescent only undergoing a reproductive diapause in which energy resources are consumed to survive rather than to produce eggs (Rossi-Stacconi et al. 2016). Crop damage in Europe predominantly occur when temperatures permit reproduction of *D. suzukii* (Hamby et al. 2013). However, different (micro)climate conditions in their habitats, e.g. altitude, determine restart of ovariole maturing and oviposition (Zerulla et al. 2015).

Although knowledge in overwintering, induction and breaking of diapause of drosophilid species is deep, e.g. for *D. montana, D. melanogaster, D. ezoana, D. triauraria, D. auraria* and *Chymomyza costata* (Saunders et al. 1989; Williams and Sokolowski, 1993; Yoshida and Kimura, 1993; Kimura and Riihimaa 1998; Koštál 2006; Salminen and Hoikkala 2013; Vaze and Helfrich-Förster 2016). Many overwintering Drosophilidae enter a reproductive diapause where the development of the ovaries stops and cold tolerance is enhanced, (Schmidt et al. 2005, Asplen et al. 2015) by using lipid and glycogen reserves (Kimura et al. 1992; Ohtsu et al. 1999).

Several studies investigated the overwintering of *D. suzukii* showed similar results. Hibernating *D. suzukii* have a improved cold hardiness, a lower enzymatic activity and higher energy reserves comparing to summer morphs (Jakobs et al. 2015; Toxopeus et al. 2016; Wallingford and Loeb 2016; Wallingford et al. 2016; Zhai et al. 2016). Moreover, studies were usually conducted with one morph type. The comparison of winter and summer morphs under identical environmental conditions is missing. Furthermore, the environmental conditions required for the induction and breaking of the winter reproductive diapause of *D. suzukii* are still unclear. A strong sensitivity of *D. suzukii* to temperature or daylength may be assumed as a morphoplastic effect of fall and/or winter conditions as reported for overwintering adults. Field studies have shown that overwintering adults are characterised by larger wings and a darker body colour than summer morphs, which is interpreted as a stronger melanisation of the cuticle because of reduced water loss rates (Rajpurohit et al. 2008, Shearer et al. 2016).

It was the aim of this work to shed light on the influence of temperature and daylength on reproductive diapause of two different *D. suzukii*-populations. The development of ovaries, egg maturation, egg deposition and adult mortality under different temperature and photoperiod conditions were assessed. Based on these results, environmental conditions responsible to induce and break reproductive diapause were deduced.

Materials and Methods

Drosophila suzukii *populations*

Two different laboratory populations of *D. suzukii* were used to carry out the experiments. One stock population was reared at longday conditions under a 16:8 hrs light:dark cycle at 22 °C and 80 % humidity (summer morph population). In the second population, individuals were reared under a 8:16 hrs light:dark cycle at 15 °C and 80 % humidity and called “winter morph (population)”. Both populations were reared for four generations and could be distinguished by morphological differences. Thus, *D. suzukii* reared under shortday conditions showed a darker coloration of the cuticle, a longer head capsule and longer wings than summer morph (Shearer et al. 2016).

A block of artificial yeast diet (modified *Cydia pomonella* diet (Bathon et al. 1991)) was offered for 2-3 days as oviposition substrate. A cotton-pad soaked with 6 % sucrose-water solution served as carbohydrate and water source. Yeast diet blocks with eggs were placed in a plastic box and kept under rearing conditions until pupation. After adult emergence, flies were released into the rearing cages.

Experimental conditions

Both populations (shortday and longday conditioned) were exposed to environmental conditions representing shifts between daylength and/or temperature, abbreviated in the following text as given in Table 1. The number of deposited eggs and the mortality of adults were observed for a 14 days period, except treatment 6. In treatment 6 adults switched from SD 10 °C to LD 20 °C after 14 days and kept for an additional 7 days (Table 1). Next, 25 females and 10 males, each ten day old, were placed in each cage per treatment. Each treatment was repeated 14 times. Animals aged 10 days were used, as at this stage of development the ovaries are fully evolved and the deposition rate reached its climax (personal observation). The influence of temperature and day length on the adapted flies should thus become more apparent.

As under rearing conditions, the adults were provided a cotton-pad soaked with 6 % sucrose-water solution and a petridish (3.5 cm diam., 10 mm height; Greiner Bio-One GmbH, Germany) filled with the artificial yeast diet served as carbohydrate, protein and water source. To avoid any potential attractive oviposition sites on the diet, the petri dishes were filled to the brim and replaced in 2-3 days intervals to prevent cracks by desiccation. Previous testing has shown that eggs have not been deposited either on the cotton-pad or into the diet, provided it was filled to the brim and another medium for oviposition was available. Therefore, blueberries were offered and accepted as exclusive oviposition substrate only when petri dishes with yeast diet were prepared accurately.

Oviposition activity

After a one-day adaptation phase of the test individuals to the respective environmental test condition, supplied with sugar water and nutrient medium only, five blueberries per cage were added as oviposition substrate. To ensure that the fruits were not spoiled by unintentional *D. suzukii* –infestation, each fruit was examined under a stereomicroscope before being exposed. Blueberries were replaced daily at the same time and examined under a stereo-microscope to assess the number of deposited eggs.

Adult mortality

In order to record adult mortality, dead individuals were removed, sexed and counted daily at the same time the blueberries were replaced, females were collected in 0.5 ml Eppendorf PCR-tubes filled with 70% ethanol and stored at 7 °C in a refrigerator until the ovaries were dissected. *D. suzukii* individuals that died during the one-day adaptation phase were replaced by 10 days old individuals, adapted separately to the same environmental condition.

Development of ovaries

In all the experiments the ovaries were dissected 14 days post start of experiment (dpe). In treatment 6, *D. suzukii* switched from shortday conditions and 10 °C to longday conditions and 20 °C after 14 days and kept for additional 7 days. Thereby *D. suzukii* which died during the first 14 days were dissected, as well as the adults at the end of the 21 day testing period.
Through a stereo-microscope (Zeiss STEMI IV) using two DUMONT-forceps for biology type 55 (neoLab Migge GmbH, Heidelberg, Germany) female *D. suzukii* were dissected and classified into three developmental stages: *immature ovaries, maturing eggs* and *mature eggs* (King *et al.*, 1956) (Fig. 1).

Data analysis

All obtained data were analysed using JMP® 11.1.1 software (SAS Institute Inc., Cary, NC, USA). Before statistical analysis, the residuals were tested for normal distribution by Shapiro-Wilk test. Based on ordinally scaled data and not following a normal distribution, the proportion of ovarian developmental stages and the percent fly mortality in the above studies were tested for the effects of treatment and conditioning as well by Kruskal-Wallis one-way test followed by a Wilcoxon Each Pair test at $\alpha = 0.05$.
Residuals of continuous data were found normally distributed. All data were subjected to an analysis of variance, procedure “Generalized Linear Models”, before further statistical analyses such as multiple comparisons of means. The respective statistical procedures and statistical core data are provided in the legends of the tables.

Results

Effect of daylength and temperature on oviposition activity

Responses to different environmental conditions were similar in the summer morph and winter morph population. At temperatures of 10 °C the lowest numbers of eggs were deposited, regardless of the daylight cycle. Irrespective of the previous adaptation of *D. suzukii*-individuals, the highest oviposition activity was found at 20 °C. Except for the combination 16:8 hrs and 20 °C, the mean number of eggs / female did not differ significantly between summer and winter morphs under the respective environmental test conditions (Fig. 2). At LD 20 °C, the highest mean number of eggs per female (28.88 ± 1.25) was deposited by summer morphs, which was significantly different to the mean (18.71 ± 0.56) calculated for the winter morph population. The highest number of eggs per female (25.99 ± 1.13) deposited by winter morphs was found under SD 20 °C, which significantly differed from the 21.43 ± 0.83 eggs / female of the individuals reared at longday conditions.

In one treatment, which had a switch after 14 days from SD 10 °C to LD 20 °C for additional 7 days, the time lapse to restart oviposition was examined. During the first 14 days, the average oviposition rate did not differ significantly between the longday and shortday adapted females (1.2 ± 0.2; and 1.43 ± 0.18 eggs per female, respectively). This corresponds to the results obtained in the treatment SD 10 °C. Two days after switching to LD 20 °C, the number of deposited eggs per female increased and another day later the oviposition rate reached the level of females kept under LD 20 °C (Fig. 2). At the end of the observation period, the mean number of eggs per female did not significantly differ between the shortday and the longday adapted females (8.91 ± 0.31 and 9.81 ± 0.31 eggs per female, respectively).

Adult Mortality

In addition to oviposition activity, mortality was assessed separately by gender. Highest mortality of females was found under longday conditions during the experiment. Most flies died in treatment LD 10 °C (26.29 % ± 2.25) (Table 2).

Daylength did not affect total mortality of male *D. suzukii* in any experimental treatment. In fact, there was a tendency to higher mortality of males descended from the summer morph population. Thus, the highest mortality of 28.10 % ± 3.21 was documented at SD 10 °C (summer morphs) (Fig. 3; Table 2).

Divergences in the total mortality within the conditioned populations could be observed (Table 2). In the summer morph population, total mortality differed significantly between LD 10 °C and LD 20 °C, and within the winter morph population, treatment LD 20 differs significantly from treatment LD 10, SD 20 and SD 10, but not from SD 15. The lowest overall mortality (10 % ± 3.0) was found for LD 20 °C conditioned adults reared under shortday conditions. The highest total mortality was observed in the groups LD 10 °C (25.31 % ± 2.2) and SD 20 °C (23.06 % ± 1.51).

In treatment 6, in which adults switched from SD 10 °C to LD 20 °C after 14 days and kept for an additional 7 days, total mortality after 14 days was similar to that in the respective treatment no. 5 (winter morphs: 20.22 ± 1.43; summer morphs: 19.78 ± 1.46). Total mortality increased to a higher rate at the end due to the longer observation period (26.33 % ± 1.67 and 26.53 % ± 1.73 for winter and summer morphs, respectively) (Fig. 3).

Development of ovaries

Independent of the initial climatic environment, the largest proportion of "immature ovaries" was observed at SD 10 °C (94.48 % ± 1.34 and 84.29 % ± 2.22 for winter and summer morphs, respectively) (Fig. 3). The smallest proportion of "immature ovaries" was determined for females of the LD 20 °C treatment, however, significant differences between the summer and the winter morph population were observed (37.69 % ± 2.07 and 6.46 % ± 1.69 for winter and summer morph females, respectively) (Fig. 4; Table 3).

For shortday conditioned females, the proportion of "immature ovaries" of *D. suzukii* females kept at LD 10 °C was significantly higher than that under SD 20 °C (72.35 % ± 2.26 and 59.36 % ± 3.91 respectively). However, these two values were not significantly different from those found for females in group SD 15 °C (68.69 % ± .3.39).

The same trend could be observed within the summer morph population, where the proportion of "immature ovaries" in females kept under LD 10 °C (74.10 % ± 2.60) and SD 20 °C (55,75 ± 2.61) were significantly different, as well.

In both conditioned populations, the proportion of immature ovaries in females exposed to the test conditions decreased with increasing temperature and was not determined by daylength (Fig. 4; Table. 3).

Females with "maturing eggs" was the smallest group within the dissected *D. suzukii*. Most of the females in this stage of ovary development were found at LD 20 °C within the winter morph population (27.77 % ± 1.86), whereas summer morph females had a significantly smaller proportion (12.25 % ± 1.90). Regardless of the conditioning, the lowest proportions of "maturing eggs" were observed under LD 10 °C, SD 10 °C and SD 20 °C (Fig. 4; Table 3).

Considering females with "mature eggs", both conditioned populations responded differently to the various treatments. Except for treatment SD 10 °C and SD 15 °C, significant differences could be observed between all other experimental conditions. The lowest percentage of "mature eggs" was observed under SD 10 °C (7.83 % ± 1.49 and 3.38 % ± 0.90, of winter and summer morph females, respectively). Most “mature eggs” (80.05 % ± 2.23) were found in treatment LD 20 °C.

The treatment, with an additional 7 days observation period showed similar results. Most females, which died during that experiment, had “immature ovaries” (winter morphs: 49.19 % ± 1.50; summer morphs: 49.34 % ± 2.97). The winter morph population showed a significant higher proportion of "maturing eggs” 22.97 % ± 1.48 of shortday conditioned individuals than the summer morph population (13.06 % ± 1.47).

Discussion

Similar to field conditions (Hamby et al. 2016; Shearer et al. 2016; Toxopeus et al. 2016; Zhai et al. 2016), phenological differences between the initial populations adapted to long- or shortday conditions could be observed. Individuals accustomed to shortday conditions and 15 °C had longer wings and tibia in comparison to *D. suzukii* acclimatized to longday conditions and 20 °C. They also had darker pigmentation of the cuticles, which is assumed to serve thermoregulation (Trullas et al. 2007; Shearer et al. 2016). The phenotypic similarity between the adults reared under laboratory conditions and those from the field indicate that the stress quality in the experiments corresponds to winter field conditions. It is assumed that the genes of winter morph adults, which are involved in DNA replication, female meiosis, and egg production are downregulated in this phenotype (Shearer et al. 2016). It may be assumed that this also leads to a reproductive diapause in female *D. suzukii*.

When studying the mean number of eggs/female, it is clear that significantly less eggs were produced at low temperature, irrespective of the previous adaptation. A temperature of 10 °C almost completely stopped egg deposition. However, in contrary to the findings by Wallingford et al. (2016), there was no significant effect of the day length on the egg deposition at low temperatures. At temperatures above 15 °C the oviposition was resumed. This suggests that between 10 °C and 15 °C *D. suzukii* changes into a phase where oviposition activity stops. This could confirm the existence of a reproductive diapause.

In treatment LD 20 °C and SD 20 °C, conditioning of *D. suzukii* affected the deposition of eggs significantly. The highest egg deposition of summer morphs was under longday conditions and 20 °C, while the winter morph population deposited most eggs under shortday conditions.

In contrast, low temperatures can abrogate adaptation to daylength and the observed low mean numbers of eggs per female did not differ significantly between winter and summer morphs. Thus, adaptation to temperature changes must be easier than an abrupt adaptation to daylength, as temperature changes are frequent and change of photoperiod is a slow-going process.

Treatment 6, in which adults were kept at SD 10 °C and brought to LD 20 °C after 14 days for additional 7 days, confirmed the presumption that a reproductive diapause takes place (Wallingford et al. 2016). Within the first 14 days of the experiment eggs were laid only very sporadically. After changing the temperature, it took 3 days to reach similar numbers of deposited eggs as at LD 20 °C. This confirms that individuals in a reproductive diapause can return to full oviposition within 72 hours under optimum environmental conditions (Toxopeus et al. 2016). Preconditioning in our experiments affected the mortality rate of the individuals if not kept under the conditions in which they were conditioned. A higher mortality in the winter morph population than in the shortday conditioned *D. suzukii*, suggests that winter morphs are less susceptible to changes in temperature and daylength (Wallingford et al. 2016). This corresponds to Zhai et al. (2016) who reported a higher survival of *D. suzukii* winter morphs at constant low temperature of 1 °C than summer morph individuals. Also, Stephens et al. (2015) documented a higher cold tolerance of winter morph adults.

Irrespective of conditioning, male mortality was higher than female mortality, which raises the question whether males are more susceptible to temperature changes and cold temperatures. A higher mortality of male *D. suzukii* during winter would not imperil population survival in spring, because females mated in fall store sperms in their spermatheca. Under favourable environmental conditions, egg production and deposition can be re-started without re-mating (Ryan et al. 2016) and a loss of males during winter can be accepted. Furthermore, gender-specific temperature tolerances have been hypothesized, where females are assumed to sustain lower temperatures better while males are more tolerant to high temperatures (Stephens et al. 2015).

Regarding the state of ovarian development, the largest proportion of examined ovaries was considered "immature" in all the treatments. The highest percentage of "immature ovaries" was observed under shortday conditions and 10 °C reared under longday conditions and decreased under higher temperatures, regardless of the conditioning. Similar results were found by Zhai et al. (2016) with the largest proportion of "immature ovaries" under shortday conditions (8h light and 16h dark) and gradually decreasing with increasing temperatures. However, the flies in our experiments were older than those used by Zhai et al. (2016) who assumed that adult *D. suzukii* respond most sensitively to the photoperiod during the first 3 days after adult emergence. They also described decreasing sensitivity up to the tenth day, beyond which the daylength does not matter anymore. In our experiments, the individuals were already 10 days old at the beginning of the experimental period. Nevertheless, significant differences between LD 10 °C and SD 10 °C could be observed within both populations, but without any effect on egg deposition.

The large proportion of "immature ovaries" in adults kept below 15 °C leads to the assumption that the interaction of daylength and temperature determines development of ovaries.

In accordance to the literature (Wallingford et al. 2016; Zhai et al. 2016), only a small proportion of "immature ovaries" was found in the winter morph population at LD 20 °C whereas the smallest proportion of "mature eggs" was found in the SD 10 °C treatment for both conditioned populations. In the winter morph population, extended daylength could not compensate the negative effect on "mature eggs" of low temperature (LD 10 °C) compared to the SD 15 °C condition, but at the low temperature 10 °C, extended daylength could increase the proportion of “mature eggs”. Furthermore, no significant increase of "mature eggs" could be observed between the SD 15 °C and the SD 20 °C treatment in the winter morph population. These results confirmed the assumption that *D. suzukii* enters a reproductive diapause at temperatures between 10 and 15 °C. These results permit the interpretation that temperature has a superior importance for ovarian development compared to daylength. A retarded ovarian development was documented also with a change from shortday to low-temperature (< 14 ° C) compared to a switch from shortday to longday conditions without temperature change (Saunders et al. 1989; Saunders and Gilbert 1990). In treatment 6 (Fig. 3; Table 3), a significant difference in the restart of ovarian development was found between the conditioned populations as revealed by the proportion of “maturing eggs” at the end of the additional observation period. This slow response of the population acclimatized to shortday conditions may be explained by the time necessary to continue ovarian development and production of “mature eggs” compared to the population accustomed to longday conditions. Comparing the number of eggs deposited and ovarian development in our experiments, temperature is of particular importance for oviposition, whereas daylength seems to determine the development of ovaries.

Considering both, daylength and temperature, as potential key stimuli to enter a reproductive diapause, contrasting theories have been made. Wallingford et al. (2016) describe the photoperiod as an important stimulus for diapause. Zhai et al. (2016) found the reproductive diapause of *D. suzukii* induced by shortened daylength, whereas Toxopeus et al. (2016) speak of a temperature-delayed development. Our results suggest rather a joint effect of both than an isolated effect of any of these factors. Referring to the proportion of "mature eggs" in the longday conditioned population, the shortened daylength (SD 20 °C) as well as the low temperature (LD 10 °C) reduced the proportion of "mature eggs". However, the combination of both factors (SD 10 °C) lead to a stronger reduction of this parameter, where the threshold temperature may be assumed between 10 and 15 °C. The combined action of reduced daylength and low temperature makes more sense than any single factor theory because both these factors indicate near winter and the end of the vegetation period. If there are no fruits for oviposition available, any further development of ovaries would only consume energy and reduce winter survival until the following spring when early fruits can be used for reproduction.

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Figures

Fig 1. Classification of the dissected female *D. suzukii* into three developmental stages: *immature ovaries, maturing eggs* and *mature eggs* (King *et al.*, 1956).

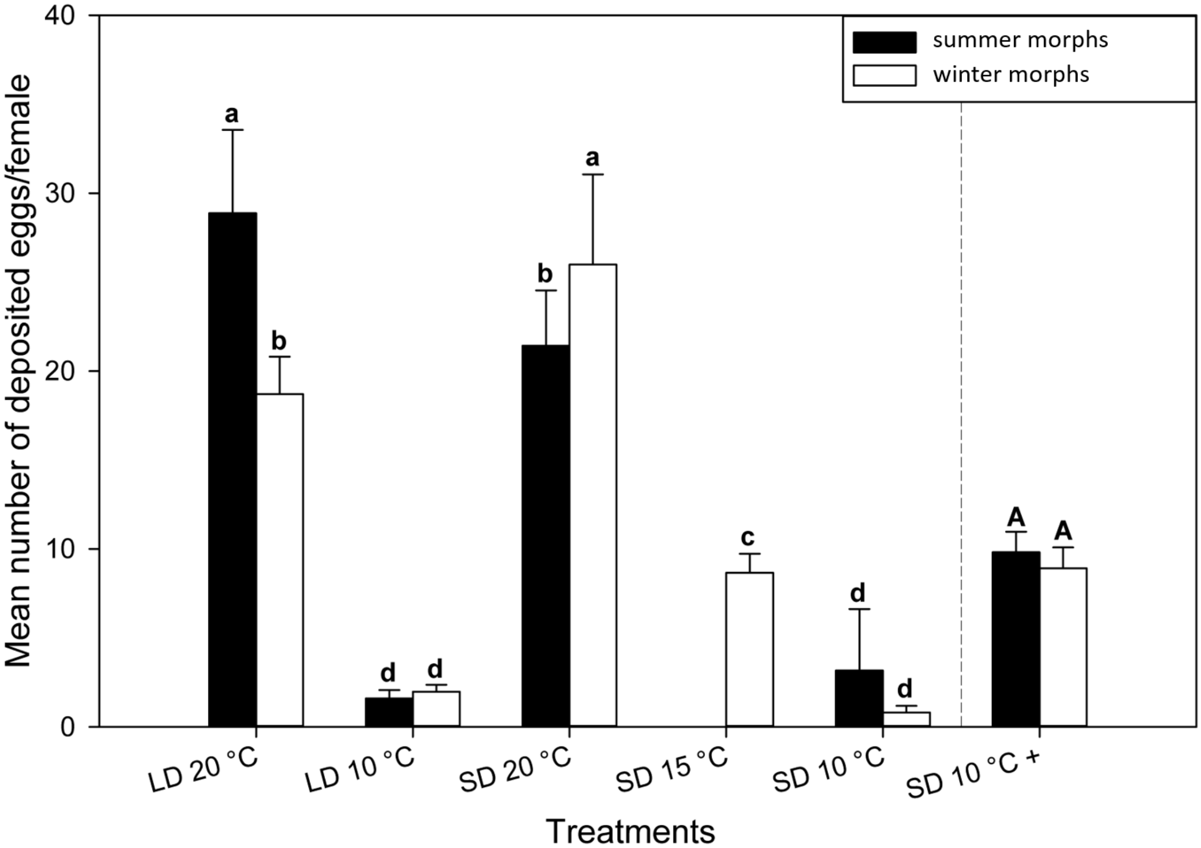

Fig 2. Mean number of deposited eggs per female *D. suzukii*.

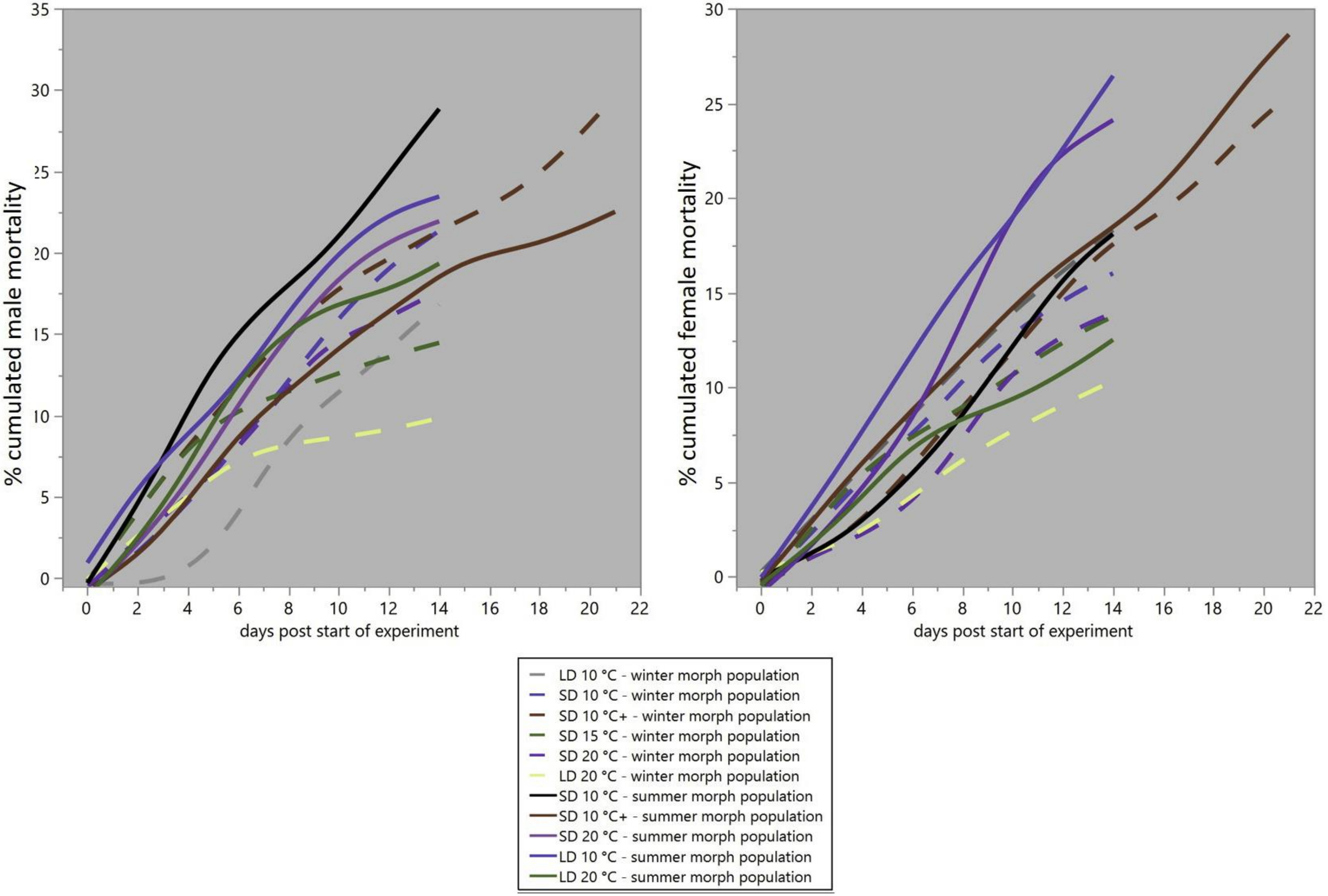

Fig 3. Percentage cumulated mortality of male and female *D. suzukii* during experiments

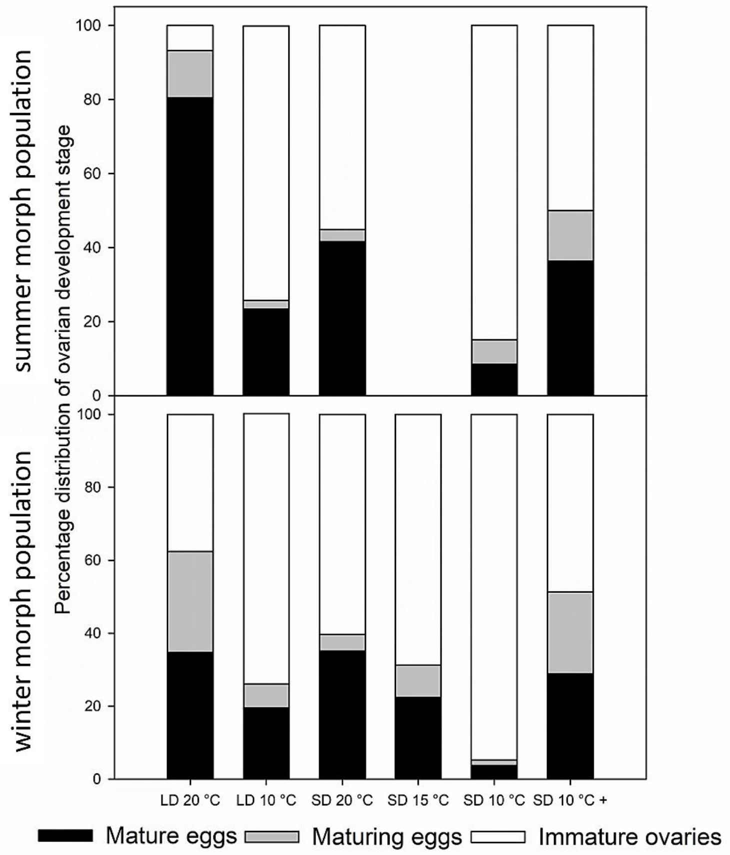

Fig 4. Percentage distribution of ovarian development stage of *D. suzukii*.

Tables

Table 1. Experimental treatments and simulated environmental conditions.

Treatment	Daylength	Temperature	Acronym	Humidity	Observation period (days post start of experiment, dpe)	Founder population
1	16L:8D	20 °C	LD 20 °C	80 %	14	summer morph
						winter morph
2	16L:8D	10 °C	LD 10 °C	80 %	14	summer morph
						winter morph
3	8L:16D	20 °C	SD 20 °C	80 %	14	summer morph
						winter morph
4	8L:16D	15 °C	SD 15 °C	80 %	14	winter morph
5	8L:16D	10 °C	SD 10 °C	80 %	14	summer morph
						winter morph
6	8L:16D	10 °C	SD 10 °C +	80 %	14	summer morph
						winter morph
	16L:8D	20 °C			7	summer morph
						winter morph

Table 2. Females, males and total mortality (%, mean ± s.e.m) (Kruskal-Wallis oneway analysis of variance, followed by Wilcoxon pairwise comparison, alpha = 0.05; different letters indicate statistically significant differences in a column).

Treatment	**winter morph population**					
	females	n	males	n	total	n
(1) LD 20 °C	10.29 ± 1.44 d	14	10.0 ± 2.97 b	14	10.20 ± 1.43 d	14
(2) LD 10 °C	19.08 ± 2.03 abc	14	16.92 ± 3.28 ab	13	18.46 ± 1.57 abc	13
(3) SD 20 °C	13.60 ± 1.02 cd	14	17.50 ± 2.16 ab	14	14.71 ± 1.16 cb	14
(4) SD 15 °C	13.43 ± 1.30 cd	14	14.29 ± 3.27 b	14	13.67 ± 0.80 cd	14
(5) SD 10 °C	15.71 ± 1.85 bcd	14	20.71 ± 3.39 ab	14	17.14 ± 0.90 bc	14
(6) SD 10 °C + (14 dpe)	18,77 ± 1.83 abc	13	23.85 ± 2.90 ab	13	20.22 ± 1.43 abc	13
(6) SD 10 °C + (21 dpe)	24.86 ± 2.15 A	14	30.0 ± 4.32 A	14	26.33 ± 1.67 A	14
	summer morph population					
	females	n	males	n	total	n
(1) LD 20 °C	12.86 ± 1.63 cd	14	20.0 ± 3.78 ab	14	14.90 ± 1.65 cd	14
(2) LD 10 °C	26.29 ± 2.25 a	14	22.86 ± 3.22 ab	14	25.31 ± 2.20 a	14
(3) SD 20 °C	23.71 ± 1.80 ab	13	21.43 ± 2.54 ab	14	23.06 ± 1.51 ab	14
(4) SD 15 °C	-	-	-	-	-	-
(5) SD 10 °C	15.81 ± 1.50 cd	14	28.10 ± 3.21 a	14	19.32 ± 1.38 abc	14
(6) SD 10 °C + (14 dpe)	19.69.0 ± 2.10 abc	14	20.0 ± 1.96 ab	13	19.78 ± 1.46 abc	13

(6) SD 10 °C + (21 dpe)	28.0 ± 2.37 A	14	22.86 ± 2.44 B	14	26.53 ± 1.73 A	14
Chi²	67.4495		26.7750		58.1799	
d.f.	10		10		10	
P	$p < 0.0001$		$p = 0.0028$		$p < 0.0001$	

Table 3. Development of ovaries (%, mean ± s.e.m); (Kruskal-Wallis oneway analysis of variance, followed by Wilcoxon pairwise comparison, alpha = 0.05; different letters indicate statistically significant differences in a column).

Treatment	**winter morph population**					
	Mature eggs	n	**Maturing eggs**	n	**Immature ovaries**	n
(1) LD 20 °C	34.75 ± 2.96 c	14	27.77 ± 1.86 a	14	37.69 ± 2.07 f	13
(2) LD 10 °C	19.34 ± 1.48 e	14	6.48 ± 1.54 ef	13	72.35 ± 2.26 c	13
(3) SD 20 °C	34.12 ± 3.29 c	13	3.70 ± 0.96 fg	13	59.36 ± 3.91 d	14
(4) SD 15 °C	22.30 ± 3.01 e	14	9.01 ± 1.28 de	14	68.69 ± 3.39 c	14
(5) SD 10 °C	3.38 ± 0.90 f	14	1.18 ± 0.62 g	13	94.48 ± 1.34 a	14
(6) SD 10 °C +	29.33 ± 1.76 cd	14	22.97 ± 1.48 b	13	49.19 ± 1.50 e	13
	summer morph population					
	Mature eggs	n	**Maturing eggs**	n	**Immature ovaries**	n
(1) LD 20 °C	80.05 ± 3.03 a	14	12.25 ± 1.90 cd	14	6.46 ± 1.69 g	13
(2) LD 10 °C	23.33 ± 2.24 de	13	2.38 ± 0.77 g	14	74.10 ± 2.60 c	13
(3) SD 20 °C	42.22 ± 2.05 b	14	3.88 ± 1.43 fg	14	55.75 ± 2.61 de	13
(4) SD 15 °C	-	-	-	-	-	-
(5) SD 10 °C	7.83 ± 1.49 f	14	6.13 ± 1.02 ef a	14	84.29 ± 2.22 b	14
(6) SD 10 °C +	35.62 ±1.28 c	13	13.06 ± 1.47 c	14	49.34 ± 2.97 e	14
Chi^2	126.5638		98.9729		128.9146	
d.f.	10		10		10	
P	$p < 0.0001$		$p < 0.0001$		$p < 0.0001$	

5. General Discussion

The survival of invasive agricultural pests in winter of new environments often determines the infestation pressure plants in the subsequent year (Keller et al. 2011; Paradis et al. 2007). Through research on overwintering strategies of alien species, adaptation and rapid propagation into the new habitats can be better understood. Therefore, the hibernation site selection and determination as well as the different factors required for induction and refraction of the diapause of *D. suzukii* were investigated in this thesis.

Using bait traps for monitoring *D. suzukii* made it possible to visualize flight activity in South Tyrol over the course of the year 2012 and 2014. The results revealed that microclimate conditions influenced the survival of an overwintering population conditions at different locations. Adult *D. suzukii* accumulated in the most favourable overwintering site (Schlossleiten), the only observation site of 30 areas where flight activity was constantly documented, and the temperature never dropped below 2 °C beneath the forest mulch.

In other localities (Barbian and Kurtatsch), no *D. suzukii* were trapped in the bait traps after the first two frost periods. It may be assumed that long exposure time to low temperatures on the ground and higher air temperatures during daytime, caused an increased mortality of the local *D. suzukii*-populations in winter 2012/2013. Therefore, the ground temperature thus appears to be important for the survival of *D. suzukii*, and mulch layer can have an important influence on the suitability of a suitable hibernation site. The leaf litter in forests can cause microclimatic differences to the surrounding environment, which helps insects during wintertime (Lee 1989). Many insects use the advantages of a mulch layer as a hibernation site, such as the larvae of *Syrphus ribesii* Linnaeus, adult *Cerotoma trifurcata* Förster, *Erynnis propertius* Scudder and Burgess and *Anthonomus grandis* Boheman (Parajulee et al. 1997; Lam and Pedigo 2000; Brown et al. 2004; Williams et al. 2012). They choose protected habitats before entering a dormancy stage to reduce their susceptibility to extreme environmental conditions (Schowalter 2016). The leaf litter can buffer large temperature fluctuations up to a certain point by decreasing high temperatures and increasing low temperatures in comparison to the environment. Therefore, the mean temperature in the mulch layer remains basically constant (Parajulee et al. 1997). An additional cover of snow above the mulch layer causes the temperature in relation to the environment not to drop below zero, which increases the survival of hibernating insects and conceivably of *D. suzukii* (Zhang 2005;

Williams et al. 2012). High humidity levels and various fungi and yeasts were found within the litter layer, which could provide an important food source for *D. suzukii* during winter (Osono and Takeda 2001; Bellutti et al. 2017).

Another important aspect could be the composition of the leaf litter. Miller and Wagner (1984), for example, reported that *Coloradia pandora* Blake only hibernate in forest areas with pine species. Thus, not only host plants are a key part for oviposition and nutrition in forests, but trees species could also play an important role, which results in an optimal composition of the leaf litter. As deciduous trees have a denser, nutritious and alkaline leaf layer (Rahman and Tsukamoto 2013), *D. suzukii* could prefer hibernation sites in deciduous or mixed forests with in closed canopy and deep leaf litter. One possibility is that *D. suzukii* leaves the hibernation habitat as soon as environmental conditions improve, which is reflected in the flight activity during winter.

Comparing the ratio of males and females caught in both winter seasons reveals comparable flight dynamics. Particularly in the months from September to December the number of captured males was higher than the number of females. After the temperatures had dropped below freezing point in January, more females were trapped when temperatures increased. These observations are consistent with the results of Vogt et al. (2012) and Stephens et al (2015), which have shown that there are differences in temperature tolerance between the sexes. Females better survive low temperatures than males and therefore occur more frequently in spring.

We were able to achieve similar results in laboratory experiments. *D. suzukii* which were conditioned under longday conditions and 20 °C showed a higher mortality than flies raised under shortday conditions and 15 °C, when temperature and/or day length were changed. This suggests that individuals acclimatized to lower temperature and shortday conditions are able to cope better with changes of environmental conditions. This is consistent with related studies of Shearer et al. (2016) and Zhai et al. (2016). They reported an increased survival of winter morph *D. suzukii* at 1 °C ambient temperature and a higher cold tolerance of winter-morphing individuals. In addition, an accumulation of glucose, trehalose, myoinositol and proline in *Drosophila montana* Stone, Griffen and Patterson females before hibernation increased the cold tolerance of the fly during the diapause (Vesala et al. 2012b). Based on these studies, it can be assumed that similar changes in the ingredients of the haemolymph also occur in *D. suzukii* and therefore the survival rate at low temperatures is higher.

We also hypothesise, due to lower temperature preferences and an increased physical activity, *D. suzukii* can spread better in spring to search for oviposition sites than flies without acclimatisation to low temperatures. According to Jakobs et al. (2015) there is a temperature difference of 0.5 °C between summer and winter morphs to enter diapause and have a faster recovering at its end. Nevertheless, the additional recording of climate data has shown that rapid temperature changes in winter could also lead to increased mortality, as *D. suzukii* exit the diapause at higher temperatures and re-enter when the temperature drops, which could cause the energy resources to deplete.

Overall, male *D. suzukii* had a higher mortality than females, independent of the condition they were raised in the climate chamber (shortday or longday conditions). This was demonstrated, by an increase of mortality due to temperature and a change in temperature preference. However, the population development is not endangered by a high mortality of male flies during the winter. Due to the spermatheca, mated females can store sperm in autumn and restart egg production under better environmental conditions in spring without being re-mated (Ryan et al. 2016).

Many female species are able to store sperm for long periods of time, sometimes longer than the duration of their breeding period or even several months and years (Giraldo-Perez et al. 2016). Sperm storage is not limited to insects, but also mammals, fish, amphibians, reptiles, birds and gastropods can store sperm (Giraldo-Perez et al. 2016; Holt and Lloyd 2010). Many of these species are inactive during unfavourable environmental conditions and hibernate during the winter, or aestivation during periods of hot or dry weather (Collet and Jarman 2001; Lehmann et al. 2010). Females may also use sperm as a food source during overwintering. It has been shown in the case of the predatory bug *Nabis rugosus* Linnaeus and *Drosophila pseudoobscura* Frolova that the amount of spermatozoa in spring depends on the nutritional status during wintertime (Collett and Jarman 2001; Roth and Reinhardt). In case *D. suzukii* does not exhaust this resource during the winter, they can use the viable sperm and fertilize eggs as soon as the first host plants bear fruit.

Thus, a rapid population development in spring is guaranteed and early ripening crops such as strawberries, blueberries or cherries are heavily infested (Grassi et al. 2011). In winter 2013/2014 and the following spring, this was demonstrated by a tenfold increase in bait catches and a massive yield loss during the entire growing season, caused by *D. suzukii*. This effect may have been exacerbated by a low mortality rate and sufficient nutrition during the mild winter and the optimal development temperatures for *D. suzukii* over the course of the year.

However, a proportion of the females showed degenerated reproductive organs in spring. We assume that *D. suzukii* first requires lipid reserves in the fat body and then absorbs eggs to cover the energy supply over winter (Ohtsu et al. 1992). Egg absorption due to the lack of food or unfavourable environmental conditions can also be found in other insects such as *Eulophu pennicornis* Nees, *Trupanea nigricornis* Coquillett and *Episyrphus balteatus* de Geer (Branquart and Hemotine 2000; Knio et al. 2007; Wakefield et al. 2010). In the absence of food over winter, this would provide *D. suzukii* with important nutrients from the resorbed eggs and make survival until spring more likely. The dissection of the female *D. suzukii* showed an increasing number of "immature ovaries" in the course of the winter, which could also indicate an absorption of eggs.

In spring, when temperature and day length increased, the number of “mature eggs” also increased. This leads to the conclusion that *D. suzukii* is able to enter a reproductive diapause, which seems to be mainly influenced by climatic conditions, nutritional status and food availability (Cini et al. 2012).

Under laboratory conditions, we were able to reproduce the results obtained in the field and draw new conclusions. It was shown that the temperature in the winter morph population (8:16 h bright: dark; 15 °C) and the summer morph population (16:8 h bright: dark; 20 °C) has a higher significance for the development of the ovaries, than the daylength. However, this effect was only significant at higher temperatures of 20 °C. At a temperature of 10 °C the oviposition almost completely stopped, irrespective the previous adaptation of *D. suzukii* individuals. Therefore, we assume that low temperatures can abrogate adaption to daylength (Wallingford et al. 2016). It is assumed that the lack of influence of the photoperiod on egg deposition could be attributed to a circadian rhythm of *D. suzukii* (Lin et al. 2014). According to Dillon et al. (2009) and Lin et al (2014) most eggs are deposited in the dark, particularly in between 20:00 and 24:00 under a 16/8 hours light/dark regimen. In addition, adapting to temperature changes has to be faster and easier than a rapid adaptation to daylength, because temperature changes are frequent, and change of photoperiod is a slow-going process in nature. This adaptability and acclimatisation capacity of *D. suzukii* leads to economic losses in areas ranging from mild subtropical growing areas to severe continental climates (Isaacs et. al 2010; Shearer et al. 2016). Thus, gradual acclimatisation can also increase the survival of *Drosophila* species, as flies are later exposed to extreme temperatures (Watson and Hoffmann 1996; Ohtsu et al. 1998; Boulétreau-Merle and Fouillet 2002; Dalton et al. 2011). Furthermore, Kelty and Lee (1998) have shown that the flies can survive lethal temperatures for short periods of time due to acclimatisation to cold climatic conditions. However, studies from Japan showed that the native *D. suzukii* had one of the lowest cold tolerances compared to other *Drosophila* species. Nevertheless, the rapid spread of *D. suzukii* in cold regions of Europe and North America confirms that the fly can adapt quickly to new habitats (Kimura 2004).

A further way of adaptation to environmental conditions are morphological changes of insects (Nylin 2013; Shearer et al. 2016). Catches by baited traps during the years 2012 - 2014 indicate that a higher trend of winter morph flies (characterized by darker pigmentation and a longer wing length) has been caught as soon as autumn changed into winter. A darker pigmentation seems to play an important role in the thermoregulation of ectothermic insects in cold environments. This is explained by the increasing absorption of ultraviolet radiation and the increased ability to rapidly heat up, even in cold climates (Harris et al., 2013; Trullas et al., 2007). Shearer et al. (2016) reported a significant increase in abdominal melanisation in *D. suzukii*, which occurred at a temperature of 10 °C and shortday conditions, compared to flies kept at 20 °C and longday conditions. Thereby, the influence of temperature seemed to have a greater influence on the melanisation, than the daylength (Shearer et al. 2016). The varying degrees of change in morphometric parameters could also be explained by different gene expression within the head and body (Shearer et al., 2016). Thus, in comparison to the head, in the body of *D. suzukii* a relatively higher number of genes is shown, which are expressed differently. In addition, the extent of different expressions seems to be much higher there (Shearer et al., 2016). In addition to the genes required for glycolysis, tricarboxylic acid cycle, electron transport chains and ATP synthase, genes responsible for morphogenesis, development and pigmentation have also been upregulated in the developed of *D. suzukii* under winter conditions, compared to summer morphs (Shearer et al., 2016). Compared to flies developed during summer, winter morphs show a significant reduction in genes associated with chromosomes, chromatin organisation, mitotic cell cycle, DNA replication and DNA repair (Shearer et al., 2016). Studies with other *Drosophila* species also suggest that there is no production of juvenile hormones in winter morph *D. suzukii* before and during the diapause, which leads to a degeneration of oocytes or follicles and ultimately reduces reproductive performance (Salminen et al. 2012). The decreased ovarian development of winter morph females therefore leads to a better adaptation to low temperatures, than individuals developed as summer morphs (Mitsui et al. 2010; Stephens et al. 2015).

This phenomenon could also be observed in the field tests as the percentage of "mature eggs" increased with higher temperatures, whereas the proportion of "immature ovaries" was highest, when female *D. suzukii* were kept at low temperatures (Zhai et al. 2016). Due to the simulated winter conditions in the laboratory the temperature needed to enter the reproductive diapause could be limited between 10 and 15 °C. At a temperature of 10 °C egg deposition almost completely stopped.

When changing from shortday conditions to warmer longday conditions, it was observed that the shortday population needed significantly longer to have the same amount of “mature eggs”, than the longday population. This slow reaction of the shortday population is explained by the time needed to continue the development of the ovaries and the production of "mature eggs" compared to the population acclimatized to longday conditions. This effect could also be demonstrated by the deposition of eggs. It took 72 hours after the change from winter to summer conditions until the amount of egg deposition increased to a normal level. In addition, the use of a temperature gradient bar in the laboratory has shown that *D. suzukii* needed approx. 25 minutes to find their preferred temperature. Thus, a rapid detection of sub-optimal or extreme temperatures ensures that the fly is protected against thermal damage or even death. Therefore, as soon as the weather conditions in nature improve, D. *suzukii* can reproduce and cause damage on wild and cultivated plants within a very short time.

Furthermore, we determined that the status of ovarian development correlated positively with deposited eggs. The highest number of egg deposition was found when *D. suzukii* were kept at 20 °C. Under these conditions most females with mature eggs were found, too. In addition, we were able to determine that fruits with a temperature of 22 °C were the most favoured for oviposition. At this temperature, the highest net reproduction rate and the highest inherent rate of population growth were found (Tochen et al. 2014). The temperature optimum of reproduction could also be reproduced very well on the egg deposition on tempered fruits. The upper threshold of 35.2 °C and the lower threshold of 11.11 °C for oviposition are close to the developmental extremes for *D. suzukii* (Kanzawa 1939; Kimura 2004; Tochen et al. 2014). Due to these findings, we assume that female *D. suzukii* oviposit only in fruits which are not exposed to harmful temperatures. This also explains the deposition of eggs in fruits that are in the shade and the increase in number of females starting oviposition in late evening hours of hot summer days (Lin et al. 2014). As soon as the fruits have cooled down to the preferential temperature, *D. suzukii* oviposit the majority (50.9 %) of its daily eggs. According to Dillon et al. (2009), this takes place between 20:00 and 24:00. It therefore appears that female *D. suzukii* deposit their eggs as a kind of brood care to appropriate temperatures by an accurate choice of oviposition sites, as this determines the speed of preimaginal development and mortality (Dillon et al. 2009). As *D. suzukii* prefers mainly dark coloured fruits, it is possible that the fruit surface may have higher temperatures due to irradiation of the sun than the ambient temperature. This could help *D. suzukii* to increase its population in spring, as the warmer fruit temperature may allow a fast preimaginal development.

Accordingly, there should be more deposited eggs on sun-facing fruits in spring and on shaded fruits during summer. This hypothesis has to be investigated deeper in future field studies. However, studies from Bergqvist et al. (2001) showed that defoliation could be a useful control measure, for example in viticulture where fruit surface temperatures of more than 37 °C were measured. According to our studies this is the upper temperature threshold for oviposition and larval development for *D. suzukii*. We therefore assume that preimaginal development is mainly affected by fruit temperature and the ambient temperature, which determine physical activity and biochemical reactions as well.

Unpublished studies revealed that *D. suzukii*, in order to preserve its physical activity, avoids extreme temperatures by colonizing different habitats during the growing season. In the native distribution area of *D. suzukii*, in Japan, other *Drosophila* species could be observed to migrate from lower to higher altitudes in summer. *Drosophila unipectinata* Duda already migrates to upper elevation in June, while *Drosophila oshimai* Choo & Nakamura, *D. suzukii* and *D. subpulchrella* migration have been documented in July (Mitsui et al. 2010; Tonina et al. 2016). It is assumed that these migrations are used as a way of avoiding summer heat in lower regions (Tonina et al. 2016). Ometto et al. (2013) reported that during summer, a higher amount of *D. suzukii* were caught at elevated areas (< 900 m a.s.l.), which is consistent with this consideration. However, these migratory flights are also a risky behaviour strategy, as insects need host plants for their populations to survive (Doucet et al. 2009). In the case of *D. suzukii*, several host plants have also been identified in higher areas, such as: *Cornus mas* Linnaeus, *Daphne mezereum* Linnaeus, *Mespilus germanica* Linnaeus, *Lonicera alpigena* Linnaeus, *Lonicera caerulea* Linnaeus, *Lonicera nigra* Linnaeus, *Lonicera xylosteum* Linnaeus, *Rubus caesius* Linnaeus, *Rubus saxatilis* Linnaeus, *Sambucus nigra* Linnaeus, *Sambucus racemosa* Linnaeus and *Sorbus torminalis* Crantz (Grassi et al. 2011; Zerulla 2013; Lee et al. 2015; Briem et al. 2016; Tonina et al. 2016). In addition, the temperatures of the host fruits in higher habitats could be more suitable for oviposition and egg development than in the hot plain.

Climate change will also change the distribution of invasive species. Masters and Norgrove (2010) assume that invasive species with short lifecycles will rapidly evolve in new habitats and overcome previous climatic boundaries. Elevated areas which used to be considered as barriers for invasive species will be eliminated by increasing temperatures in these zones, so that insects will be able to move into adjacent areas (Masters and Norgrove 2010).

Unpublished data also showed that bait catches of *D. suzukii* increased in the winter months in lower habitats. Therefore, first crops were threatened in early spring in these lower areas. However, according to Mitsui et al. (2010), other *Drosophila* species which infest unripe and/or rotten fruits would not undergo such extensive movements between lower and higher elevations.

In our studies we were able to determine that temperature is of particular importance for oviposition and preimaginal development. In addition to the temperature, however, daylength seems to influence the development of ovaries. For entering a reproductive diapause, we consider that temperature and daylength are the most important key stimuli for *D. suzukii*. In laboratory tests we were able to show that the combination of cold temperatures and shortday conditions led to a stronger reduction of "mature eggs" than these factors individually. The combined effect of reduced day length and low temperatures is more meaningful than the theory of Wallingford et al. (2016), Zhai et al. (2016) or Toxopeus et al. (2016), which assumes that individual factors are responsible for the induction of the diapause. We believe that both environmental factors indicate near winter and the end of the vegetation period. If there are no fruits for oviposition, any further ovarian development would drain energy and reduce survival of female *D. suzukii* over winter.

Our knowledge of the key temperatures affecting oviposition and development of ovaries as well as the temperature adaptation capacity of *D. suzukii* can be used for better prediction of infestation. Through important additional information and data for modelling population dynamics could be provided. Especially early ripening fruit varieties might be protected better by developing long-term strategies of population dynamics management. However, more precise predictions on infections of other crops could also be made during the course of the year.

Another major and important point in the control of *D. suzukii* will be the characterization and identification of overwintering sites. This can help to facilitate the development of targeted control strategies to delay population dynamics in the following season. For this purpose, further research has to focus on the development of environmentally friendly control methods in order to control *D. suzukii* in the overwintering sites during winter.

Outlook

Although the research results presented in this paper have provided important insights into the hibernation behaviour of *D. suzukii*, many questions remain unanswered and further research is necessary in connection with the invasion of *D. suzukii* into the intensive fruit and wine growing in the northern hemisphere.

The advancing climate change can cause *D. suzukii* to spread even further into northern regions of Europe and the United States of America and colonize elevated areas within the affected countries. With increasing numbers of weather extremes, *D. suzukii* has to adapt to these changing conditions in order to ensure the population's survival. This adaptation can be achieved by morphological or physiological changes or by changing behavioural characteristics and must be investigated in further experiments. Based on the analysis of the components of haemolymph, it is important to determine the accumulation of glucose, trehalose, myoinositol and proline while entering the diapause. Moreover, a focus should be set on hormonal changes (juvenile hormones and ecdysteroids) before, during and after the diapause. Therefore, it should be possible to limit the diapause inducing environmental conditions even more precisely, as this could determine the exact induction/refraction of the diapause.

Based on the results of the present thesis, it seems very useful to understand the role and importance of ecology of *D. suzukii*, for example, the importance of environmental factors on the phenotypic expression of this pest. In order to determine in which development phase *D. suzukii* can perceive environmental factors and adapt its phenotype accordingly, studies should be carried out on this research topic.

Furthermore, it should be investigated which habitats are used by *D. suzukii* for hibernation in forests. If certain hibernation sites could be identified, an effective and environmentally friendly control would be possible already before vegetation period begins.

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7. Summary

Drosophila suzukii (Matsumura) was introduced to southern Europe and the United States of America in 2008 through fruit imports from Southeast Asia and spread in the following years all over Europe, as well as South and North America. *D. suzukii* is a polyphagous pest which infests fruits of soft-skinned wild and cultivated plants. In contrast to the well-known *D. melanogaster*, healthy and ripe fruits are preferred. The infestation is caused by female *D. suzukii* who damage the fruit skin to deposit eggs underneath with the help of their serrated ovipositor. The feeding of hatching larvae and secondary infections, which can easily penetrate through the damaged fruit, can lead to complete yield losses. A short reproductive period, a large range of host plants and infestation of the fruits, shortly before harvesting makes it extremely difficult to control the pest. Due to comparatively late infestation, the possible period of application during ripening and harvesting is limited. The same applies to the frequency of application of insecticides at this time. Possible residues on the harvested products also carry the risk of rejection of the fruit on the market. Furthermore, an incomplete knowledge of the biology, especially overwintering biology under European environmental conditions makes an effective control of this invasive pest extremely difficult.

Therefore, the main research topics are the induction and refraction of the postulated diapause, the detection of any possible hibernation sites and the influence of temperature on the oviposition behaviour of *D. suzukii*.

Based on field experiments it could be shown that successful wintering could probably only take place in forest areas. After freezing, the forest was the only place where *D. suzukii* could resume its flight activity at warmer temperatures. It has also been confirmed that female flies are more resistant to temperatures below freezing than male flies. Accordingly, after a frost period, hardly any male *D. suzukii* were caught in bait traps. Similar results have also been obtained in laboratory tests, showing that *D. suzukii* adapted to low temperatures and shortday conditions had lower temperature preferences and a decreased mortality after changing environmental conditions. It can therefore be assumed that overwintering *D. suzukii* can spread better in spring due to a lower temperature preference and a higher physical activity under cold environmental conditions than flies without adaptation to winter conditions.

Bait traps were also used to determine the developmental status of the ovaries by dissecting the abdomens of weekly captured *D. suzukii*. This enabled a correlation between microclimatic conditions of individual habitats and the reproductive status of females to be established. In winter, the majority of female *D. suzukii* had “immature ovaries”, whereas in the summer most females had “mature eggs” in their abdomen. For this reason, it can be assumed that *D. suzukii* entered a reproductive diapause, which is apparently influenced by winter climatic conditions, nutritional status and the availability of food.

In addition, it was found that the developmental status of ovaries correlates positively with oviposition. Laboratory tests were carried out to determine the highest number of egg depositions at 20 °C. Most females with “mature eggs” were also documented under these simulated conditions. We detected that the preferred surface temperature for egg depositing was very similar to the preferred ambient temperature of *D. suzukii*. Most of the eggs were deposited on fruits with a surface temperature of 22 °C. At this temperature, the highest net reproductive rate and intrinsic rate of population increase was found, too. Temperatures below 15 °C and above 35 °C were not preferred, which represented the thresholds for a successful development of *D. suzukii*. Temperatures between 10 and 15 °C and shortday conditions were the most important key stimuli for entering the reproductive diapause. Therefore, temperature had a stronger influence on oviposition behaviour than daylength. A complete disruption of diapause occurred at higher temperatures (20 °C) and longday conditions after 72 hours.

The data presented in this work on the possibility of adapting *D. suzukii* to environmental conditions and key temperatures, which influence the development of ovaries and egg deposition, can provide an important contribution to the development of prediction and population dynamics models and can be used for long-term control strategies against *D. suzukii*.

According to current knowledge and observations, overwintering is obviously a critical period for the survival of the populations. Therefore, the characterisation and identification of additional hibernation sites is of great importance. There, a targeted and environmentally friendly control of *D. suzukii* populations could be particularly efficient.

8. Zusammenfassung

Die Kirschessigfliege (*Drosophila suzukii*, Matsumura) wurde 2008 durch Fruchtimporte aus dem südost-asiatischen Raum nach Südeuropa und den USA eingeschleppt und verbreitete sich in den Folgejahren in ganz Europa, sowie in Süd- und Nordamerika. Bei *D. suzukii* handelt es sich um einen polyphagen Schadorganismus, welcher Früchte von weichschaligen Wild- und Kulturpflanzen befällt. Dabei werden, im Gegensatz zur bekannten *D. melanogaster* gesunde und reife Früchte bevorzugt. Der Schaden entsteht nur durch die weiblichen *D. suzukii*, die mithilfe ihres gezähnten Ovipositor die Fruchthaut beschädigen, um darunter ihre Eier abzulegen. Durch den Fraß der schlüpfenden Larven und nachfolgenden Sekundärinfektionen, die durch die beschädigte Frucht leicht hervorgerufen werden können, kann es zu vollständigen Ertragsverlusten kommen. Die kurze Reproduktionszeit, das große Wirtspflanzenspektrum und ein Befall der Früchte kurz vor der Ernte, macht eine Kontrolle des Schädlings äußerst schwierig. Durch einen vergleichsweisen späten Befall ist der mögliche Anwendungszeitraum während der Reife- und Erntephase beschränkt. Gleiches gilt für die Anwendungshäufigkeit von Insektiziden in dieser Zeit. Dadurch hervorgerufene mögliche Rückstände auf dem Erntegut bergen das Risiko der Ablehnung der Früchte auf dem Markt. Eine unvollständige Kenntnis der Biologie, besonders der Überwinterungsbiologie unter europäischen Umweltbedingungen macht eine effektive Bekämpfung des invasiven Schädlings zudem äußerst schwierig.

Der Schwerpunkt dieser Arbeit lag deshalb auf Untersuchungen zur Frage, wie die Winterruhe von *D. suzukii* induziert und gebrochen wird, der Aufdeckung möglicher Überwinterungsplätze und dem Einfluss der Temperatur auf das Eiablageverhalten der Kirschessigfliege.

Anhand von Freilandversuchen konnte gezeigt werden, dass eine erfolgreiche Überwinterung wahrscheinlich nur in Waldgebieten stattfinden kann. Nach Frostereignissen war der Wald der einzige Standort, an dem *D. suzukii* bei nachfolgend wieder wärmeren Temperaturen ihre Flugaktivität wieder aufnehmen konnten. Zudem wurde bestätigt, dass weibliche Kirschessigfliegen resistenter gegenüber Temperaturen unter dem Gefrierpunkt sind, als männliche. Demnach wurden nach einem Frost kaum noch männliche *D. suzukii* in Köderfallen gefangen. Ähnliche Ergebnisse konnten auch in Laborversuchen erzielt werden, die zudem zeigten, dass Tiere, die an kühle Temperaturen und Kurztagbedingungen angepasst wurden eine niedrigere Temperaturpräferenz und Mortalität bei wechselnden Umweltbedingungen hatten. Es ist daher davon auszugehen, dass sich überwinternde *D. suzukii* durch eine niedrigere Temperaturpräferenz und eine höhere körperliche Aktivität bei kühlen Umweltbedingungen im Frühjahr besser ausbreiten kann, als Tiere ohne eine derartige Akklimatisation an Winterbedingungen. Mithilfe von Köderfallen wurde wöchentlich der Entwicklungszustand der Eierstöcke durch das Sezieren der Abdomen der gefangenen Kirschessigfliegen festgestellt. Es konnte eine Korrelation zwischen mikroklimatischen Bedingungen einzelner Standorte und dem Reproduktionszustand der Weibchen festgestellt werden. In den Wintermonaten wies der größte Teil der Kirschessigfliegen unreife Ovarien auf, wohingegen im Sommer die meisten Weibchen reife Eier in ihren Abdomen trugen. Es ist deshalb anzunehmen, dass *D. suzukii* in eine reproduktive Diapause eintritt, die offenbar durch winterliche klimatische Bedingungen, dem Ernährungszustand und die Verfügbarkeit von Nahrung beeinflusst wird.

Darüber hinaus konnte festgestellt werden, dass der Entwicklungszustand der Ovarien positiv mit den Eiablagen korreliert. In Laborversuchen konnte die höchste Eiablage bei 20 °C erfasst werden. Bei dieser Temperatur wurden auch die meisten Weibchen mit reifen Eiern dokumentiert. Außerdem konnte festgestellt werden, dass die präferierte Oberflächen-temperatur für Eiablagen sehr nah an der bevorzugten Umgebungstemperatur lag. Die meisten Eier wurden auf Früchte mit einer Temperatur von 22 °C abgelegt. Bei dieser Temperatur wurde auch die höchste Reproduktionsrate und die schnellste Wachstumsrate der Individuen festgestellt. Temperaturen unter 15 und über 35 °C wurden nicht präferiert, was gleichzeitig auch die Grenzwerte für eine erfolgreiche Entwicklung der Kirschessigfliegen darstellte. Temperaturen zwischen 10 und 15 °C und Kurztagbedingungen waren die wichtigsten Schlüsselreize für den Eintritt in die reproduktive Diapause. Dabei hatte die Temperatur einen stärkeren Einfluss auf das Eiablageverhalten der Tiere, als die Tageslänge. Eine vollständige Brechung der Diapause erfolgte bei höheren Temperaturen (20 °C) und Langtagbedingungen nach einem Zeitraum von 72 Stunden.

Die in dieser Arbeit beschriebenen Daten über die Möglichkeit der Adaptation von *D. suzukii* an Umweltbedingungen und Schlüsseltemperaturen, welche die Entwicklung der Ovarien und die Eiablage beeinflussen, können einen wichtigen Beitrag für die Entwicklung von Befallsprognose- und Populationsdynamikmodellen liefern und für langfristige Kontroll-strategien genutzt werden. Da die Überwinterung nach bisherigen Erkenntnissen und Beobachtungen offensichtlich einen kritischen Zeitraum für das Überleben der Populationen darstellt, ist die Charakterisierung und Identifizierung von weiteren Überwinterungsplätzen von großer Bedeutung. Eine gezielte und umweltfreundliche Eindämmung der *D. suzukii*-Population könnte dort besonders effizient sein.

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54. Österreichische Pflanzenschutztage; 27.11 – 28.11.2013; Stift Ossiach, Österreich; Title: *Untersuchungen zur Ei-Entwicklung in den Ovarien der Kirschessigfliege* (Drosophila suzukii).

IOBC Working Group "Integrated Plant Protection in Fruit Crops" Subgroup "Soft Fruits"; 26.05. – 28.05.2014; Vigalzano di Pergine Valsugana, Italy; Title: *About the overwintering ability of* Drosophila suzukii *in South Tyrol.*

Beerenseminar 2014; Agroscope, SOV-FUS; 20.11. – 21.11.2014; Ittlingen, Switzerland; Title: *Überwinterung von* Drosophila suzukii *– ein Blick über die Grenze.*

7. BayWa Weinbausymposium; 08.01.2015; Heilbronn; Title: *Eine Fliege sieht rot! Verhaltens- und Ernährungsbiologie der Kirschessigfliege* (Drosophila suzukii).

Actualités Phytosanitaires/Pourriture acide et Drosophiles; 19.01.2015; Saint Hippolyte, France; Title: *Überwinterung der Kirschessigfliege und deren Eiablageverhalten im Frühjahr.*

Workshop: Strategien zum Umgang mit der Kirschessigfliege im Ökologischen Obst- und Weinbau; 27.01.2015; LTZ Augustenberg; Title: *Untersuchungen zur Überwinterung der Kirschessigfliege.*

Erfahrungsaustausch zur Kirschessigfliege; DOW AgroScrience; 05.02.2015; Deidesheim; Title: *Verhaltensbiologie der Kirschessigfliege.*

2. Öko-Beerenobsttag NRW; 19.02.2015; Münster-Wolbeck; Title: *Kirschessigfliege – Biologie und Lebensweise der* Drosophila suzukii.

Entomologentagung 2015; 02.03. – 05.03.2015; Frankfurt/M.; Title: *Untersuchungen zur Ei-Entwicklung in den Ovarien der Kirschessigfliege* (Drosophila suzukii).

LIEBEGGer Tag der Spezialkulturen; 23.04.2015; Lupfig, Schweiz; Title: *Neues zur Biologie der Kirschessigfliege.*

Fellbacher Weintreff; 11.05.2015; Fellbach; Title: *Informationen zum Befall und zur Kontrolle der Kirschessigfliege* (Drosophila suzukii).

4. AG Kirschessigfliege; 08.12.2015; Erfurt: Title: *Untersuchungen zum Eiablageverhalten der Kirschessigfliege (*D. suzukii*) auf unterschiedlichen Rebsorten.*

16. **Bioland-Weinbautagung**; 11.01. – 13.01.2016; Nals, Italy; Title: *Kirschessigfliege: Aktuelle Erkenntnisse aus Deutschland.*

Fachtagung zum Ökologischen Obstbau; 29.01.2016; Jork; Title: *Kirschessigfliege: Aktueller Stand und Forschungsergebnisse.*

17th International Conference on Organic Fruit Growing; 16.02.2016; Stuttgart; Title*: Field performance of different lures for* Drosophila suzukii.

1. Doktorandentag Hohenheim; 06.10.2016; Stuttgart-Hohenheim; Title*: A new invasive pest in Europe: Overwintering biology of* Drosophila suzukii.

5. AG Kirschessigfliege; 06.12. – 07.12.2016; Weinsberg; Title: *Einfluss der Umgebungs- und Fruchttemperatur auf das Eiablageverhalten von* D. suzukii.

8. BayWa Weinbausymposium; 12.01.2017; Heilbronn; Title: *„Eine staubige Angelegenheit“ – alternative Bekämpfungsmöglichkeiten gegen die Kirschessigfliege*.

Ökologische Beerenobsttagung; 03.02.2017; Weinsberg; Title: *Stand der Forschung zur Kirschessigfliege*.

Entomologentagung 2017; 15.05.2017; Weihenstephan; Title: *Effect of mineral dusts on oviposition behavior of the spotted wing drosophila*.

6. AG Kirschessigfliege; 05.12 – 06.12.2017; Bad Kreuznach; Title: *Auswirkungen unterschiedlicher Temperaturen und Tageslängen auf die Induktion und Brechung der Diapause und den Zustand der Ovarien von* D. suzukii.

Florian Zerulla

Stuttgart, November 18

Eidesstattliche Versicherung

gemäß § 8 Absatz 2 der Promotionsordnung der Universität Hohenheim zum Dr.sc.agr.

1. Bei der eingereichten Dissertation zum Thema

„ Overwintering and reproduction biology of *Drosophila suzukii*, Matsumura (Diptera: Drosophilidae)"

handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Florian Zerulla

Florian Zerulla
Stuttgart, November 18

Eidesstattliche Versicherung

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Die entsprechenden Strafvorschriften sind in § 156 StGB (falsche Versicherung an Eides Statt) und in § 161 StGB (Fahrlässiger Falscheid, fahrlässige falsche Versicherung an Eides Statt) wiedergegeben.

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Wer vor einer zur Abnahme einer Versicherung an Eides Statt zuständigen Behörde eine solche Versicherung falsch abgibt oder unter Berufung auf eine solche Versicherung falsch aussagt, wird mit Freiheitsstrafe bis zu drei Jahren oder mit Geldstrafe bestraft.

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Abs. 2: Straflosigkeit tritt ein, wenn der Täter die falsche Angabe rechtzeitig berichtigt. Die Vorschriften des § 158 Absätze 2 und 3 gelten entsprechend.

Ich habe die Belehrung zur Eidesstattlichen Versicherung zur Kenntnis genommen.

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Florian Zerulla

Stuttgart, November 18