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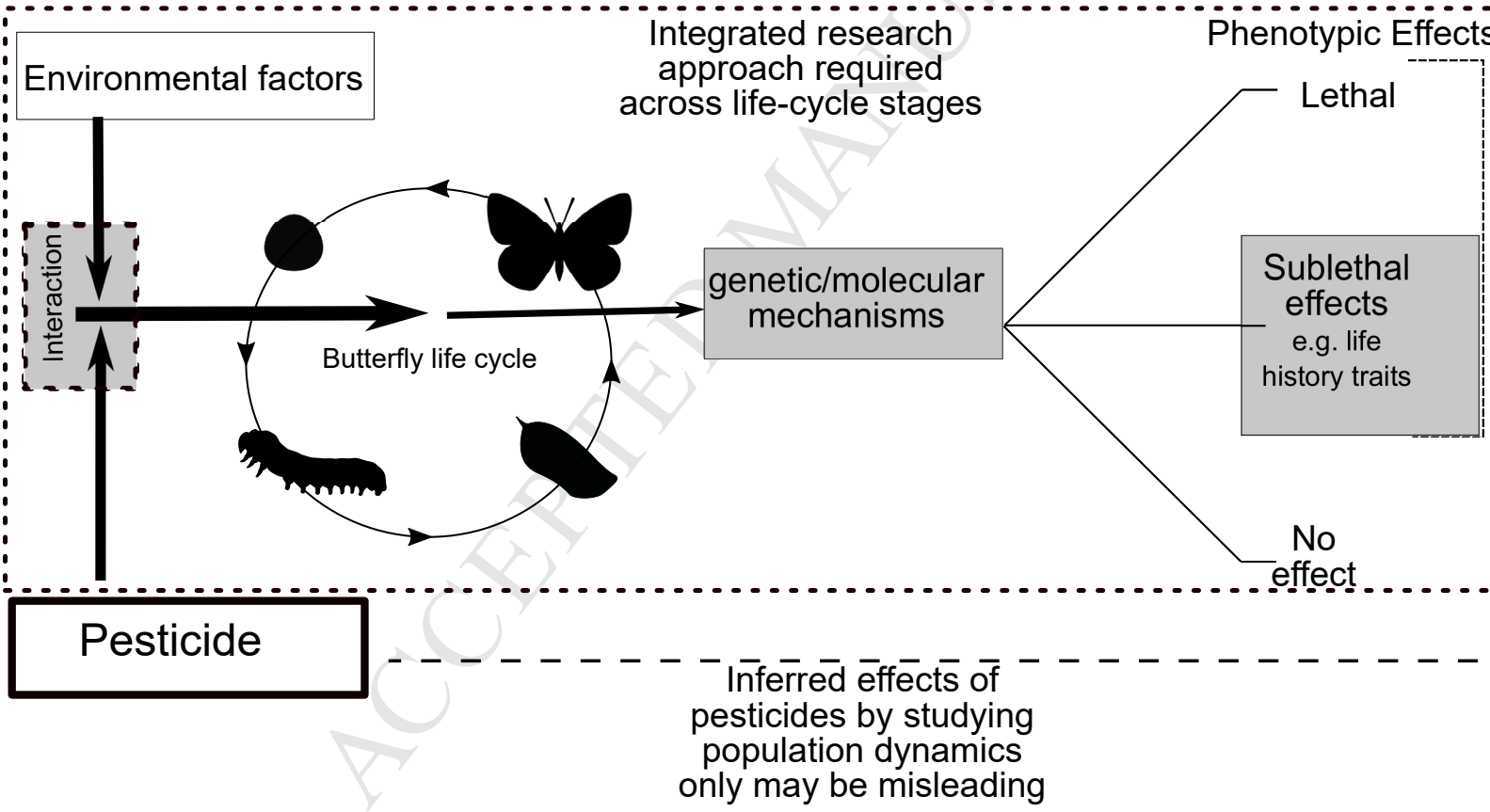

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The effects of insecticides on butterflies – a review

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ABSTRACT

Pesticides, in particular insecticides, can be very beneficial but have also been found to have harmful side effects on non-target insects. Butterflies play an important role in ecosystems, are well monitored and are recognised as good indicators of environmental health. The amount of information already known about butterfly ecology and the increased availability of genomes make them a very valuable model for the study of non-target effects of pesticide usage. The effects of pesticides are not simply linear, but complex through their interactions with a large variety of biotic and abiotic factors. Furthermore, these effects manifest themselves at a variety of levels, from the molecular to metapopulation level. Research should therefore aim to dissect these complex effects at a number of levels, but as we discuss in this review, this is seldom if ever done in butterflies. We suggest that in order dissect the complex effects of pesticides on butterflies we need to integrate detailed molecular studies, including characterising sequence variability of relevant target genes, with more classical evolutionary ecology; from direct toxicity tests on individual larvae in the laboratory to field studies that consider the potentiation of pesticides by ecologically relevant environmental biotic and abiotic stressors. Such integration would better inform population-level responses across broad geographical scales and provide more in-depth information about the non-target impacts of pesticides.

Short summary: We propose an integrated research approach, from the molecular level up, to fully gauge the effects of pesticides on non-target butterfly species

Key words: butterflies; population dynamics; non-target effects; pesticide; bio pesticide

1. Introduction

1.1 Non-target effects pesticides

There is no doubt that pesticides can be enormously beneficial in both agriculture and preventive medicine, for example to increase (the quality of) crop yields, to maintain healthy livestock and to prevent the spread of diseases (Oerke, 2006; Cooper and Dobson, 2007; Aktar *et al.*, 2009; Benelli and Mehlhorn, 2016; Guedes *et al.*, 2016). However, due care is needed for their use in an effective manner. Not only do we need to carefully establish the mode of action of pesticides, but also the effects of pesticides on both their intended targets and non-target species. It is clear that where innocent bystanders of pesticides find their natural habitat replaced or reduced by agricultural practices they are doubly affected (Potts *et al.*, 2016). One such group of insects are Lepidoptera which may comprise good indicator species for the non-target impacts of pesticides. Our relationship with Lepidoptera is a complex one. On the one hand they are the focus of considerable conservation efforts, predominantly butterflies (Brereton *et al.*, 2011; Potts *et al.*, 2016), but on the other hand 70% of agricultural pests are Lepidoptera, in particular many moth species and a few butterflies. Various studies on pest moth species have identified genes that could be targeted for pest control, either through pesticides, or genome editing techniques (Guan *et al.*, 2018). While there is a substantial body of literature on pesticide use and effects on moths (e.g. Shakeel *et al.* (2017)), a comprehensive overview for butterflies is lacking (Pisa *et al.*, 2015). Furthermore, although numerous studies have addressed the effects of land use *per se* on butterfly population dynamics and life-history strategies, very few have taken pesticide use into account (Lebeau *et al.*, 2016; Hallmann *et al.*, 2017; Malcolm, 2018). In this review we will therefore provide a comprehensive overview of what is known about the effects of pesticide use on butterflies, provide novel insights, highlights gaps in our knowledge, and propose future directions of study. Finally, it is hoped that although the focus will be on

butterflies, extrapolation will be possible to those benign moth species that have seen their numbers reduced, not least due to indiscriminate effects of pesticides (Fox, 2012).

Benefits of using pesticides in agriculture range from nutritional health and/or increased diversity of viable crops, to more derived secondary benefits such as a reduced migration by humans to cities and a better educated population (Cooper and Dobson, 2007; Aktar *et al.*, 2009). On the other hand, the increased use of pesticides can also result in harmful side-effects for wildlife (Boutin *et al.*, 1999; Bell *et al.*, 2001; Mineau, 2005). While such negative impacts of modern, intensive agriculture on biodiversity have been widely recognised, the contribution that agricultural pesticides make to this overall impact has largely been neglected (Gibbs *et al.*, 2009; Gilburn *et al.*, 2015). Insecticides are one of the biggest classes of pesticides used in the world (Aktar *et al.*, 2009), and this review reflects that insecticides are also the class of pesticides predominatly investigated in butterflies. Although insecticides are produced as a pest preventative method, the vast spectrum of their toxicity inadvertently leads to the suppression of non-target insects and organisms inhabiting the same niche or environment. Affected, non-target organisms might include pollinators, natural predators and parasites (Johansen, 1977).

The main focus of research on non-target pesticide effects has been the European honey bee (*Apis melligera)* (Sanchez-Bayo and Goka, 2014). The honey bee is the most economically valuable pollinator of crop monocultures and their absence could cause a decrease in yield of up to 90% in some crops (Southwick and Southwick, 1992; Winfree *et al.*, 2007; Arena and Sgolastra, 2014). In recent years many (managed) bee colonies suddenly died over winter, through a phenomena named Colony Collapse Disorder (CCD) (vanEngelsdorp *et al.*, 2009). The cause of CCD is unknown and is probably the result of a complex interaction between multiple factors. One of the factors implicated in CCD are pesticides, especially neonicotinoids (Ratnieks and Carreck, 2010; van der Sluijs *et al.*, 2013;

Lu *et al.*, 2014; Pisa *et al.*, 2015). Neonicotinoids are the most used class of pesticides in the world. They are widely applied as seed dressing and work systemically throughout the plant. Neonicotinoids mimic the acetylcholine neurotransmitter and are highly neurotoxic to insects (Goulson, 2013; van der Sluijs *et al.*, 2013; Crossthwaite *et al.*, 2017). The indication of their role in CCD caused the European Union to ban three pesticides in the class of neonicotinoids in 2013, namely clothianidin, thiamethoxam and imidacloprid (European-Commission, 2013). The observation of CCD and the consequent neonicotinoid ban renewed and intensified the interest and research into the (non-target) effects of neonicotinoids in particular and pesticides in general (e.g. Pisa *et al.* (2015); Woodcock *et al.* (2016); Wood and Goulson (2017); Woodcock *et al.* (2017))

Although honey bees are cheap, versatile, easy to manage and create their own economically valuable product they are not the most effective pollinator for a lot of crops (Klein *et al.*, 2007). Furthermore, honey bees are not the only non-target species affected. A recent review by Pisa *et al.* (2015) assessing the impact of pesticides on non-target species, identified a need for studies investigating the effect of pesticides on Lepidoptera, in particular butterflies (see also Wood and Goulson (2017)).

1.2 Butterflies as models for non-target effects of pesticides

Butterflies play an important role in ecosystems as plant pollinators (Feber *et al.*, 1997; Potts *et al.*, 2016) and as prey for other organisms (Strong *et al.*, 2000). Well-known to the general public, they are well monitored, recognised as indicators of environmental health (Whitworth *et al.*, 2018) and as such they have been used to measure impact of factors such as climate change (Schweiger *et al.*, 2012) and landscape fragmentation (Scriven *et al.*, 2017). Comparatively, their ecology and abundance is much better known than any other

invertebrate taxa (New, 1997). This allows the possibility to investigate the impact of pesticides across a large ecological range (Fontaine *et al.*, 2016). Butterfly species diversity and abundance has already been shown to be influenced by landscape complexity and type of farming (Rundlöf and Smith, 2006), quality of habitat (Pocewicz *et al.*, 2009) and habitat management (Marini *et al.*, 2009). Obviously some butterfly species are agricultural pests, such as the cabbage white species (*Pieris* sp.), but nothing like the scale and species diversity observed for moths (Feber *et al.*, 1997). Understanding butterflies' sensitivity and responses to pesticide exposure more fully might help assess the overall risk of pesticide use (Pisa *et al.*, 2015). The availability of genomic data for an ever-increasing number of butterfly species allows one to investigate the observed sensitivity and responses at the underlying molecular level (Shen *et al.*, 2016; Liu *et al.*, 2018), but also how they may adapt to agricultural environments (Sikkink *et al.*, 2017). Research at the level of such integration in butterflies is far behind that of moths, and thus the detailed studies on pesticide development, usage and effects on pest moths can provide valuable starting points for such an approach (Troczka *et al.*, 2017)

The habitat of many butterfly species consists of hedgerows or the fragmented areas between arable lands (Warren *et al.*, 2001; Krauss *et al.*, 2003). Butterflies can therefore come into contact with pesticide treated plants and areas through foraging or translocation. Butterflies inhabiting hedgerows are susceptible to spray drift from insecticides (Davis *et al.*, 1991a; 1991b; Çilgi and Jepson, 1995; Kjær *et al.*, 2014). Numbers of widespread butterflies on monitored farm land have declined by 58% between 2000 and 2009 (Brereton *et al.*, 2011), and a number of species are under threat. Some pesticides are applied in the form of a coating around seeds, this coating leaves a residue in the soil, and if water-soluble this residue can enter the ground water (Bonmatin *et al.*, 2015; Schaafsma *et al.*, 2015). Uptake from soil and soil water by non-target plants, particularly those in hedgerows and field

margins is another potential route of (sub)lethal exposure in non-target species (Goulson, 2013). Butterflies that engage in mud puddling behaviour can also be exposed to pesticide residues or run-off in soil water (Still *et al.*, 2015). Pesticides, such as neonicotinoids, that have systemic properties can translocate to pollen, nectar and guttation droplets, and become other potential routes of exposure (van der Sluijs *et al.*, 2013). For example, via plant surfaces, as butterflies may collect honey dew/sap from trunks and leaves. However, little is known about the presence of pesticides in honey dew, but Corke (1999) suggested that 15 different species of honey dew/sap feeding UK butterfly species may have been negatively affected by exposure to particulate air pollution via this route. Therefore, there is the potential for these butterfly species to also be adversely affected by exposure to systemic pesticides, such as neonicotinoids, via honey dew/sap feeding. Adult feeding also has the potential to result in transovarial transport of pesticides from mothers to offspring, including bio pesticides (Paula *et al.*, 2014). Insect growth regulators such as juvenile hormone analogues and chitin synthesis inhibitors are particularly amenable to transovarial transport (Campbell *et al.*, 2016). However, much more work is required to explore the full range of potential routes by which butterflies may be exposed to pesticides in nature.

2. Data source and study selection

Here we provide a comprehensive review of research on the effects of pesticides on butterflies. The number of published studies on pesticide use and effects on butterflies is very small in comparison to that of moths, and we have set out to review every single study in this overview, making it therefore unique in its depth. We have identified three main approaches to pesticide research on butterflies, each of which will be discussed in turn in this review. The first approach largely investigates the effects of pesticides on butterflies through the study of population trends. These studies use butterfly abundance and species richness data and

compare these across places or times with different levels of pesticide usage. The second approach consists of field tests whereby researchers actively modify the use of pesticides in a (semi) natural environment. The third, and possibly the most used approach, is the examination of the direct effects of pesticides on all, or a selection of, stages in the butterfly lifecycle.

3 Effects of pesticide use on butterflies

3.1 Changes in butterfly abundance and species richness in response to pesticides

To our knowledge, eight studies have explicitly examined population trends to determine the non-target effects of pesticides on butterflies, usually as part of a population dynamics modelling approach (Feber *et al.*, 1997; Salvato, 2001; Feber *et al.*, 2007; Brittain *et al.*, 2010; Pekin, 2013; Gilburn *et al.*, 2015; Muratet and Fontaine, 2015; Forister *et al.*, 2016). More often than not, studies merely infer the contribution of pesticide use on population trends (Malcolm, 2018). Six of these studies compared similar areas with different levels of pesticide usage and determined the differences in butterfly abundance and/or species richness between those areas (Feber *et al.*, 1997; Salvato, 2001; Feber *et al.*, 2007; Brittain *et al.*, 2010; Pekin, 2013; Muratet and Fontaine, 2015). The approach taken by the two remaining studies, Gilburn *et al.* (2015) and Forister *et al.* (2016), differed from the other six. These two studies did not compare locations with different levels of pesticide use at the same point in time, but used time as a variable in their models and compared butterfly abundance before and after the introduction of neonicotinoids. These studies and the approaches used will be examined in more detail throughout this section.

Pekin (2013) used a large scale dataset, not focusing on absolute abundance of butterflies in the analyses, but rather on the number of butterfly species. This study found that variation in Turkish butterfly species composition was largely explained by the combination of agricultural chemical use, especially pesticides, with climate and land-cover variables. The significance of these variables varied per Turkish province, and thus location. Muratet and Fontaine (2015) used a large-scale dataset, collected by the public which considered pesticide use and butterfly abundance in their gardens. Pesticides, especially insecticides and herbicides were found to have a negative impact on butterfly abundance. This study examined an aspect of pesticide use often overlooked; the non-industrial use of pesticides. Although these effects might be smaller, gardens can be very important refuges for butterflies (Fontaine *et al.*, 2016).

The other four studies compared sets of similar land types where the biggest difference across treatments was the amount of pesticide used. Feber *et al.* (2007) and Feber *et al.* (1997) used paired sets of neighbouring organic and non-organic farms to compare butterfly abundance. Both of these studies found that irrespective of the type of crop present, non-pest butterfly species were more abundant on organic farms, especially in the uncropped field margins. Brittain *et al.* (2010) used a pair of intensively farmed basins in Italy versus a nature reserve and compared whether intensively farmed land with high pesticide use had lower species richness than the nature reserve, which had negligible amounts of pesticide use. This study found that at the regional scale, butterfly species richness was lower in the intensely farmed basin with the high pesticide loads. Salvato (2001) surveyed 9 transects in South Florida and Lower Florida Keys for adult and larval densities of three species of butterflies; *Anaea troglodyte, Strymon acis bartrami* and *Hersperia meskei*. All pesticide treatment areas were compared against controls; areas where insecticide applications are restricted. In most cases, there was a lower butterfly density in the sprayed locations

compared to the control sites. Larval density seems to be highest in unsprayed transects, and increased in transects that ceased insecticide application.

Finally, as mentioned previously, the studies of Gilburn *et al.* (2015) and Forister *et al.* (2016) differ from the other six studies in the approach they used to study the impact of pesticides on butterfly abundance. Gilburn *et al.* (2015) used UK-wide abundance data of 17 widespread resident butterfly species that routinely breed in any field or field margin habitats for their analysis. They modelled data from 1985 to 2012 and their model included a whole range of current and previous year weather measurements such as mean temperature and rainfall during the seasons, as well as the previous year's population index for each species and previous year's pesticide use. A strong negative correlation between butterfly population size and the amount of neonicotinoids used in previous years was observed. In 1998 neonicotinoid use in the UK exceeded 100,000 hectares for the first time. To examine the impact of this increase in neonicotinoid usage on butterfly abundance, Gilburn et al. (2015) split their data set up into two different time periods, one from 1985 to 1998 and one from 1998 to 2012. Remarkably, when the same model was applied to analyse variation in butterfly abundance across these two-time periods, the abundance of widespread butterflies showed a significant increase in the first -1985 to 1998- dataset, and a decrease in the second -1998 to 2012. These data suggest that increased usage of neonicotinoid pesticides may correlate with a decline in the abundance of 17 widespread UK butterfly species.

Forister *et al.* (2016) used a somewhat similar approach to the Gilburn *et al.* (2015) study but over a smaller geographical scale using longitudinal data from 4 North Californian locations experiencing butterfly declines since the late 1990's . In each of the locations the presence of 67 butterfly species was monitored on a bi-weekly basis for 40 years. A negative relationship between neonicotinoid use and annual variation in butterfly species observations was readily detectable, while controlling for land use and other factors. Furthermore, smaller-

bodied butterfly species and those with fewer generations per annum showed more severe declines in response to neonicotinoid exposure.

Even though these eight studies used a wide variety of different experimental and statistical approaches to examine the response of butterfly species over a range of spatial and temporal scales, a similar trend was reported by all; increased pesticide levels lead to reductions in butterfly abundance or species richness. The trends reported in these articles are in line with general expectations i.e. pesticide use can have detrimental non-target effects on butterflies. However, these studies do highlight some other important and interesting factors that require further consideration. One of these is consideration of how much non-industrial use of pesticides might affect vulnerable species, especially in places like gardens which are increasingly being used in urbanised landscapes by many butterfly species as habitat patches that provide essential resources such as nectar sources and host plants for oviposition (Fontaine *et al.*, 2016). More detailed research into this area would be very valuable (Muratet and Fontaine, 2015), especially because butterfly abundance and species richness have been shown to be negatively correlated with pesticide use in gardens (Fontaine *et al.*, 2016).

Studies examining population trends to determine the non-target effects of pesticides on butterflies are very informative as the effects of pesticides are complex, and looking at the real-world effects can give vital insight into the actual scale of the effect. These studies also provide an opportunity to explore the impact of indirect effects, for example through complex interaction and by reducing the number of suitable host plants. Although factors, such as weather, interacting with pesticide use should be taken into account, this is not always done, through a lack of power in the dataset. A vast number of butterfly species utilise host plants commonly considered to be weeds, which may be targeted by herbicides (Malcolm, 2018). Whilst crops may be genetically modified to develop herbicide resistance, other plants may be affected by herbicide spray drifts. This reduction in host plant availability or quality may

also lead to reduction in butterfly abundance without having any direct toxicity effects on butterflies (Smart *et al.*, 2000). In Feber *et al.* (2007) this idea was explored by comparing differences in botanical compositions between the organic farms and conventional farms. Although no difference in grass and forb species between organic and conventional field boundaries was found, there may be differences in the abundances of particular nectar sources and host plants, which could impact butterfly population dynamics.

3.2 Field studies

Studies addressing the effects pesticides on butterflies, as well as genes involved, in a field context are based on butterflies that are considered pest species, including *Pieris brassicae* (cabbage butterfly), *Pieris rapae* (small cabbage white butterfly), *Pieris napi* (green-veined white), *Virochola livia* (pomegranate butterfly), and *Papilio demoleus* (lemon butterfly) (Liu *et al.*, 2018). Such studies do not examine effects on non-target butterfly species. However, they do give a good insight into the actual field efficacy and thus the potential level of harmfulness to butterflies in general, particularly because the method of application, as well types of areas where some pesticides are applied suggest the potential for affecting non-target butterflies.

First, we will discuss studies focussing on *P. brassicae* as a target species. Davis *et al.* (1991b) compared the pesticide sensitivity of larvae from three butterfly species in the lab and established that *P. brassicae* as tested by Sinha *et al.* (1990) showed higher sensitivity to the following tested insecticides; Dimethoate, Phosalone, Fenitrothion and Diflubenzeron. This led them to conclude that *P. brassicae* might be a good indicator species for the effects of pesticides on butterflies in general (Davis *et al.*, 1991b). Subsequently both *P. brassicae* and *P. napi* larvae were exposed to the same spray drift at field-realistic concentrations,

which again showed *P. brassicae* to be the more sensitive species to the pesticide diflubenzuron, another insecticide. The molecular mechanisms or other reasons why *P. brassicae* seems to be more sensitive to pesticides than the other tested species were not addressed. Muthukumar *et al.* (2007) and Thakur and Deka (1997) combined, tested 19 different pesticides for their efficacy to kill or deter *P. brassicae* larvae. All of these 19 treatments had a significant effect, greatly reducing the number of larvae. Thakur and Deka (1997) mention six pesticides (deltamethrin, cypermethrin, malathion, fenitrothion, endosulphan and monocrotophos) with a field efficacy higher than 90%, and one, fenvalerate, had a field efficacy of 100%. These numbers indicate that these pesticides are highly toxic to *P. brassicae*, and potentially toxic to other butterfly species too. As these pesticides are applied by spray there is a high possibility of drift and thus contact with non-target butterflies.

Another frequently investigated pest species is the pomegranate butterfly (*V. livia*), in countries including Egypt, Cyprus and Jordan (Obeidat and Akkawi, 2002; Kahramanoglu and Usanmaz, 2013; Abd-Ella, 2015). *Virachola livia* lay their eggs on fruit, and after hatching the larvae bore into the fruit, causing crop damage. In contrast to the aforementioned *P. brassicae* studies, larval mortality levels were not measured. Instead, the reduction of fruit infestation and fruit damage after pesticide application was studied. Although a reduction in fruit damage was observed, the mechanism underlying this reduction is unknown, and it is unclear whether it is due to pesticides acting as an oviposition deterrent, or due to the pesticides directly killing eggs or larvae. A closer look into the mechanisms of crop protection could help to indicate the possible non-target toxicity effects on other butterflies and insects. These studies indicate that a wide range of pesticides may have high field toxicity to butterflies, suggesting that numerous, different pesticides are highly likely to have non-target effects.

In addition to chemical pesticides there are also bio pesticides. Bio pesticides are natural occurring substances that control pests (Copping and Menn, 2000). Fungi and a bacterium called *Bacillus thuringiensis* (*Bt*) are commonly used as bio pesticides but other kinds of bio pesticides such as plant extracts are also used (Copping and Menn, 2000). Use of *Bt* as a biopesticide, including *Bt*-transgenic plants resistant to lepidopteran pests, appears effective against *P. brassicae* and *P. rapae* but less so for *P. demoleus* (Zafar *et al.*, 2002; Narayanamma and Savithri, 2003; Muthukumar *et al.*, 2007). However, this strategy is not without risks for non-target species through ingestion of GM *Bt* pollen (Manachini *et al.*, 2018) or through transmission of *Bt* toxins to offspring via eggs (Paula *et al.*, 2014; Lang and Otto, 2015). Treatment with fungi is again effective against *P. rapae* but not against *P. demoleus,* with fungi being even less effective against *P. demoleus* than *Bt* (Zafar *et al.*, 2002; Narayanamma and Savithri, 2003). The use of organisms that cause disease as bio pesticides raise additional questions of possible negative non-target effects such as how long can they persist in the environment? Can they be transmitted between individuals, and how far can these infections be carried (Tilquin *et al.*, 2008; Duchet *et al.*, 2014)? These types of questions are particularly relevant for *Bt* as this bio pesticide is used extensively in aerial sprays for control of forest defoliators such as gypsy moth, *Lymantria dispar*, and western spruce budworm, *Choristoneura occidentalis*. Although the short half-life of *Bt* in the field is believed to minimise its impact on non-target Lepidoptera, some studies have demonstrated that it can be toxic to some non-target butterflies, such as *Papilio glaucus* for at least 30 days after the spray (Johnson *et al.*, 1995), and transgenerational effects have been reported (Paula *et al.*, 2014).

Non-target field studies can be divided into two categories; studies that look at the effects of pesticide spray drift (Davis *et al.*, 1991a; Davis *et al.*, 1991b; Davis *et al.*, 1993; Davis *et al.*, 1994; de Jong and van der Nagel, 1994; Zhong *et al.*, 2010) and studies that

adjust the application of pesticides, mainly to leave the crop edges and hedgerows unsprayed (Rands and Sotherton, 1986; Dover *et al.*, 1990; de Snoo *et al.*, 1998). The latter category of studies examined how pesticides affect butterfly abundance in hedgerows, which are often considered as a safe-haven for butterflies, in particular when agricultural fields are turned into monocultures without suitable host plants. In their review, Dover and Sparks (2000) discuss the importance of hedgerows in detail; a total of 39 of the 61 UK resident or regular butterfly species have been recorded in hedgerows, making hedgerows an important biotope for conservation. Hedgerows and their grassy surroundings can provide larval host plants, shelter, flowering nectar sources and a corridor system for dispersal for adult butterflies (Fry and Robson, 1994; Longley and Sotherton, 1997). The severity of the impact of pesticides on each of the 39 hedgerow-associated species is likely to depend on the degree by which they utilise this important biotope. For example, some species can be totally supported by hedgerows, other species use them to breed, and some species only fly in from other core habitats to bask, feed or use them as transport corridors. As such it may be expected that species with a higher association with hedgerows may be more greatly impacted by the non-target effects of pesticides. More studies would be required however to confirm this (Dover and Sparks, 2000).

Rands and Sotherton (1986) compared a fully-sprayed plot of arable land with one that had the field edges left unsprayed with pesticides. The number of butterflies observed between May and August was significantly higher in the latter (868 vs. 297). Of the 17 species that were observed more than once, 13 were more abundant in the unsprayed plot. Similarly, Dover *et al.* (1990) monitored butterflies in each treatment across years 1995 to 1997 on 14 UK conservation headlands each of which fell into one of four types, short hedges, tall hedges, wood edges or railway embankments. The conservation headlands were selectively sprayed with some pesticides including an insecticide, although which insecticide

364 was used and in what dose was not reported. The four types of headlands also had
365 significantly fewer butterflies in the field areas with fully sprayed headlands. Furthermore,
366 the pierids *Anthocharis cardarnines, P. napi* and *P. rapae* all managed to lay eggs in the
367 conservation headland on their host plants *Sinapis arvensis L.* and *Brassica napus,* be it in
368 low densities. A similar study conducted in the Netherlands also reported fewer butterflies in
369 sprayed margins than in unsprayed margins. It did depend both on the crop type and the year
370 examined (Snoo *et al.*, 1998). It can be hypothesised that the favourable effects on butterfly
371 abundance in the unsprayed margins were mainly due to the greater availability of flowering
372 plants but could not be tested with the data from Snoo et al (1998). Such hedgerow studies
373 also provide some insights not only into indirect effects of pesticides but also into potential
374 interaction effects with other factors. An example includes the effects of herbicides and
375 fertilisers on butterflies and their associated hostplants (Longley and Sotherton, 1997).

376 Spray drift is named as one of the main sources of non-target butterfly exposure to
377 pesticides, as pesticides drift over from fields of arable land to areas with higher number of
378 resources for butterflies such as hedgerows, wildflower patches or even nearby nature
379 reserves (Sinha *et al.*, 1990; Zhong *et al.*, 2010). Quite a few studies examine ground-level
380 spraying effects on butterflies (Davis *et al.*, 1991a, b, 1993, 1994; de Jong and van der Nagel,
381 1994), while Zhong *et al.* (2010) addressed the impacts of aerial ultra-low volume spraying of
382 Naled on the Miami blue butterfly in Florida. Naled is used to target mosquitoes and a small
383 droplet of Naled created by the ultra-low volume spraying does not settle quickly and is
384 capable of drifting extended distances both in and out of the target area. The Miami blue
385 butterfly (*Cyclargus thomasi bethunebakeri*) is endemic to Florida and has been in serious
386 decline. In addition to habitat loss, climate change and a handful of other factors, the use of
387 the aerial application of Naled has been indicated as a possible contributory factor in their
388 decline. Naled was found to negatively affect late instar Miami blue larvae at the

concentration found in the target zone, but not at the concentrations found in the spray drift zones (Zhong *et al.*, 2010). However, whether the concentrations of Naled found in the spray drift zones affects other larval instars or life stages of these butterflies requires further work (Zhong *et al.*, 2010).

However, it was found that even at low wind levels pesticides could drift and cause high mortality to *P. brassicae* larvae up to 24 metres away from the spray site (studies reported in table 1 and Supplementary File). For example, Davis *et al.* (1994) monitored 2-day-old *P. brassicae* were placed on plants at different distances from a field sprayed with cypermethrin, recording a higher mortality of larvae for three days after spraying. They included an examination of how landscape features, especially hedgerows, could influence the spread of pesticides by spray drift, by acting as a barrier, and concluded that hedges may provide a sheltered area immediately behind the hedge, but as the distance from the hedge increases, larval mortality increases again minimising the shelter effect of the hedge. de Jong and van der Nagel (1994) also placed *P. brassicae* at different distances from a plot of land sprayed with diflubenzuron. In this study the LD-50 was established at only 0.16% of the sprayed dose, and the drift from the application was at a sufficiently high concentration to still cause larval mortality. As expected, the closer the larvae were to the sprayed area the higher were the mortality levels. These studies indicate that pesticide spray drift has the potential to cause serious mortality in butterfly species over considerable distances from the sprayed area, and that landscape features, such as hedges, are ineffective barriers to spray drift.

3.3 Direct toxicity effects of pesticides on butterflies

Here, we were interested in determining how many different butterfly species have been used in direct toxicity tests, which pesticides have been tested on butterflies, in what dose and which butterfly life stages have been examined. For example, recent studies on *P. rapae* dissecting the sensitivity and response to pesticides at the molecular level (e.g. identification of relevant genes) do so in a life-stage specific way (Liu *et al.*, 2017; Liu *et al.*, 2018).

In total, 22 species of butterflies were used in direct toxicity tests of pesticides (Table 1). It should be noted that these were all insecticides. Ten of these species were exposed to such pesticides in both the larval and adult stages and one species, *P. brassicae,* was used in egg and larval stage. Three species, *Ascia monusta, Bicyclus anynana and Dryas julia,* were only tested in the adult stage and the remaining eight species were only tested in the larval stage. The number of studies published per species is highly variable, ranging from a single study for the majority of species studied, to 12 different studies on *P. brassicae.* As mentioned earlier in this review, *P. brassicae* has been demonstrated to be more sensitive to pesticides than some of the other species studied, and has therefore been suggested to be a good model species for examining the impact of pesticides on butterfly pest species (Davis *et al.*, 1991b). This may explain why the majority of studies examining effects of pesticides are on this species. In total, we found 31 studies that examined the direct effects of pesticide exposure on butterflies (Table 1). The majority of these studies performed direct toxicity tests on the larval stage (n= 26 studies), a few have considered the adult stage (n = 8 studies), but hardly any studies have examined the impact of pesticide usage in the egg stage (n = 2) and none examined the pupal stages in butterflies (Table 1). Few studies have considered the sub-lethal effects of pesticides through the different stages of the life cycle to the adult stage, or considered potential for transgenerational effects (i.e. the transfer of the effects of pesticides from parents to offspring). Although the larval stage is probably the most economically

damaging phase of the butterfly life cycle, and thus the most suitable part of the life cycle to target for pest control, it would be valuable to examine how pesticides impact other life stages to provide further insights into the non-target and sub-lethal effects of pesticides on butterfly populations.

In the studies detailed in Supplementary table 1, butterflies have been directly exposed to pesticides (i.e. insecticides) using 3 main methods; 1) direct physical exposure, bringing a droplet of pesticide of a specific concentration straight on to, often the thorax, of the larvae or adult butterfly, 2) using a similar method to 1 in which the egg, caterpillar or adult butterfly was sprayed with, or otherwise physically exposed, to a pesticide and 3) larvae are exposed to food plants treated with a pesticide. Additionally, in two studies the larvae were exposed via a plant grown on pesticide treated soil (Krischik *et al.*, 2015; Basley and Goulson, 2018).

A wide range of pesticides have been tested for their toxic effects on butterflies, and 19 of these studies report a LD-50 for that pesticide under their tested conditions (Supplementary table 1). Although these values give a rough indication of the toxicity of each particular pesticide for butterflies, there are a number of factors that may affect the generality of these findings. First, the response to any given pesticide is likely to be very species-specific. The study by Hoang *et al.* (2011) provides a good example of why it is important to consider species-specific responses to pesticides. They exposed 5th instar larvae of four different butterfly species to the pesticide Naled. The range of LD-50 at 24 hours after exposure lies between 0.19 μg/g for *Anartia jatrophae* and 10.82 μg/g for *Vanessa cardui*, which means that a fifth instar *A. jatrophe* caterpillar is almost 57 times more sensitive to Naled than a fifth instar *V. cardui* caterpillar. This is a difference that cannot solely be explained by a difference in larval size as *V. cardui* 5th instar larvae (0.553±0.05 g) are only 1.3 times heavier than *A. jatrophe* 5th instar larvae (0.425±0.012 g).

Second, the response to a pesticide is highly dependent on the life stage of the butterfly examined; a first instar caterpillar might be more sensitive than the fourth instar caterpillar of the same species (reviewed in Wood and Goulson (2017)). This effect is well demonstrated by the results of Eliazar and Emmel (1991), showing that different stages of the life cycle have different levels of sensitivity to pesticides and that these patterns are not predictable and depend on the pesticide examined. Fourth instar larvae of *Papilio cresphontes* have an LD-50 of 193.01 μg/g for Fenthion and an LD-50 of 62.463μg/g for Malathion whilst fifth instar larvae of the same species have LD-50s of 41.1 μg/g and 128.455 μg/g respectively. For both pesticides, the sensitivity of *P. cresphontes* depended on the instar of the larva but for Fenthion the sensitivity decreased, while for Malathion it increased with larval age. Additionally, Davis *et al.* (1993) shows that even a couple of days can have a big difference on the sensitivity of larvae to pesticides. Two-day old *P. brassicae* larvae have an LD-50 of 1.521 μg/g when Triazophos is topically applied, while four-day old larvae have an LD-50 of 3.283 μg/g. In the moth *Spodoptera frugiperda*, increased tolerance to the pesticides methomul, diazinon and permethrin with larval age was associated with increased midgut aldrin epoxidase and gluthathione S-transferase activity (Yu *et al.*, 2015). However, more studies would be required to determine whether similar mechanisms are responsible for the age-specific variation in insecticide susceptibility observed in butterfly larvae. The mechanisms underlying these subtle changes in sensitivity and differences in trends between pesticides require further investigation. This could provide valuable insights into the modes of action of pesticides and determine when and how pesticides are most effective.

Lastly, the method of application could potentially have a large influence on the effect of pesticides. Dhingra *et al.* (2008) exposed third instar of *P. brassicae* to cypermethrin in two different ways; spraying the larvae with pesticide versus feeding the larvae with leaves dipped in the cypermethrin. The larvae had an LD-50 of 9.0 μg/ml when fed with leaves

dipped in cypermethrin, versus an LD-50 of 11.6 µg/ml LD-50 when they were directly sprayed. Such differences in sensitivity could have major effects in the field.

In order to test what effects pesticides may have, field-realistic doses should be used as was done when testing the effects of the neonicotinoid clothianidin on the development and survival of *Polyommatus icarus* (see Supplementary table 1; Basley and Goulson (2018)). Reduced larval growth and elevated mortality levels were detected, but ideally the interaction between pesticide use and other factors (e.g. climatic variables and host plant quality) should be studied to get a more realistic indication of the potential effect of pesticides in the environment on multiple aspects of the butterfly development.

In conclusion, based on the values found in these studies alone it is difficult to estimate on the harmfulness of a specific pesticide to non-target butterflies, because the effects of the pesticide are likely to be influenced by the environmental context and the method of application used. To estimate the actual field harmfulness, we would need much more detailed knowledge about normal field doses the butterflies are exposed to, at what stages butterflies are most likely to be exposed, for how long or how often they will be exposed and what is the most likely exposure method that will be used. Additionally, looking only at lethal doses prevents the investigation of other negative sub-lethal effects of pesticides which could impact fitness-related traits and butterfly abundance at the population level. Sub-lethal effects of pesticides on beneficial arthropods have been found to include effects on neurophysiology, larval development, moulting, adult longevity, immunology, fecundity, sex ratio, mobility, navigation and orientation, feeding behaviour, oviposition behaviour and learning (Desneux *et al.*, 2007; Belzunces *et al.*, 2012; de França *et al.*, 2017). The compounding effect of these factors might have a negative impact on butterfly abundance even if the initial pesticide exposure is not lethal. Of the 31 studies detailed in Supplementary table 1, only 12 measured the sub-lethal impacts of pesticides on butterflies.

11 used larval traits (e.g. larval size, development time etc.), and 3 used adult traits (e.g. longevity, fecundity etc.) as a measure of sub-lethal effects. A very small number (n=4) measured behavioural traits, namely feeding adverse behaviour (Tan, 1981; Xu *et al.*, 2008; Vattikonda *et al.*, 2015) or egg laying choice (Oberhauser *et al.*, 2006). None of the studies to date have examined sub-lethal effects of pesticides on neurophysiology or immunology in butterflies. Consideration of whole-organism sub-lethal effects would be very valuable to provide more realistic estimates of the longer-term impact of pesticides on butterfly abundance. Synergistic effects may also play an important role in nature. Synergy occurs when the effect of a combination of stressors is higher than the sum of the effect of each stressor alone (van der Sluijs *et al.*, 2013). The impacts of immunity on moths are already known for three pesticide classes; botanical insecticides, inorganic insecticides and insect growth regulators (James and Xu (2012) provide an extensive review of mechanisms by which pesticides affect insect immunity). Synergy for pesticides and pathogen infection therefore has a high potential in butterflies and requires further investigation.

4. Defence mechanisms against pesticide exposure

As mentioned in the previous section, there is some evidence for differences in sensitivity to pesticides both within and across life stages. We will discuss the possible ways that butterflies may be able to defend themselves against exposure to pesticides across life stages.

There are numerous different classes of pesticides specifically designed to disrupt one or more different processes to cause insect mortality such as; the nervous system (e.g. organophosphates, carbonates, pyrethroids, avermectins, neonicotinoids), energy production (e.g. amidinolydrazone, pyrrole), cuticle production (insect growth regulators e.g.

methoprene, pyriproxyfen, fenoxycarb) and water balance (boric acid, silica aerogels, diatomaceous earth) (Sparks and Nauen, 2015). Some insecticides are very selectively toxic to Lepidopteran pests such as the bisacylhydrazine insect growth regulators Tebufenozide and RH-2485, both of which induce lethal larval moults via interaction with ecdysteroid receptor proteins (Dhadialla *et al.*, 1998). Other insect growth regulators such as aromatic non-terpenoidal insecticides like pyriproxfen (which mimic the action of juvenile hormone) are toxic to a broad spectrum of insects, including Lepidoptera, during their embryonic, last larval or reproductive stages (Dhadialla *et al.*, 1998). The potential for non-target effects of these insecticides on butterflies is therefore very high, particularly because these types of modern insect growth regulators have been specifically designed to have a much greater metabolic and environmental stability so that they are better suited for use in agriculture (Dhadialla *et al.*, 1998). Currently, it is unknown why bisacylhydrazines have such a high lepidopteran pest specificity and aromatic non-terpenoidal insecticides do not, especially because most insects use ecdysteroid and/or juvenile hormone as moulting hormones (Dhadialla *et al.*, 1998). When first introduced for pest management it was widely believed that insects would not be able to develop resistance mechanisms to molecules that mimic their own hormones, but this has not proved to be the case (see Dhadialla *et al.* (1998) for an extensive review of the insecticidal, ecotoxiological and mode of action of bisacylhydrazines and non-terpenoidal insecticides). More work is required, however, to explore the non-target impacts of insect growth regulators on butterflies and the capacity of butterflies to defend themselves against this class of insecticides.

Resistance to chemical insecticides can be caused by one or more of the following mechanisms; behavioural avoidance, reduced permeability (e.g. through the cuticle), increased metabolic detoxification or decreased sensitivity of the target (Heckel, 2009; Lilly

560 *et al.*, 2016), with the latter two mechanisms being the most commonly encountered (Heckel,
561 2009).

562 If butterflies are able to recognise the presence of toxins visually, via olfaction or via
563 contact, behaviours adopted by adult butterflies during oviposition or by larvae during
564 feeding can aid in toxic plant avoidance (see e.g. Després *et al*., 2007) for an extensive
565 review of the evolutionary ecology of insect resistance to plant alleolochemicals). For
566 example, larvae of the butterfly *D. plexipus* feed on plants with secretory canals, and the
567 larvae cut trenches to depressurise the canals and reduce toxic exudation at their feeding site
568 (called canal trenching behaviour, Després *et al.*, 2007). Female butterflies are able to detect
569 plant defensive compounds during oviposition, and the genes involved appear not only to
570 evolve very rapidly, but also duplicate readily with the resulting paralogs increasing the
571 capacity of ovipositing females to detect a larger variety of (complex) plant compounds
572 (Briscoe *et al.*, 2013; Engsontia *et al.*, 2014). It has been suggested that evolution in response
573 to host plant defences may serve as a preadaptation to surviving exposure to modern synthetic
574 insecticides (Després *et al.*, 2007; Heckel, 2009). In particular, there is potential for metabolic
575 resistance to insecticides with a chemical structure similar to some of the plant-produced
576 defensive chemicals, such as pyrethroids and neonicotinoids (Després *et al.*, 2007; Heckel,
577 2009). However, more work, and a greater integration of classical resistance studies with
578 chemical ecology would be required to examine this further, but the long co-evolutionary
579 history of insect-plant interactions in Lepidoptera would make them ideal models for such
580 studies (Heckel, 2009).

581 Reduced permeability can occur via multiple routes including enhanced expression of
582 metabolic resistance mechanisms in the integument, increased presence of binding proteins,
583 lipids and/or sclerotisation that trap insecticides, a measurably thicker cuticle, or a
584 combination of some or all of these mechanisms together (Lilly *et al.* (2016) and references

585 therein). Only one study to date has demonstrated a role for reduced penetration in conferring
586 resistance to a pesticide in Lepidoptera; changes in cuticular composition in response to DDT
587 in the tobacco budworm (Vinson and Law, 1971). In other insects, reduced permeability has
588 been implicated in insecticide-resistance to pyrethrin, organophosphates, carbonates and
589 organochlorines, but ordinarily by itself, reduced penetration does not provide a high level of
590 resistance and typically is only found when other mechanisms are present (Lilly *et al.*, 2016,
591 and references therein). However, insect eggs are adaptively structured to provide a barrier
592 that protects the embryo against penetration by environmental stressors, and are therefore
593 considered the most difficult life stage to kill with pesticides (Campbell *et al.*, 2016).
594 Campbell *et al.* (2016) have provided an extremely comprehensive review of the mechanisms
595 by which insect embryos are protected against pesticides via both reduced penetration
596 through egg shell barriers, and by enzymatic resistance. Lepidopteran eggs have been shown
597 to be susceptible to the following ovicidal insecticides; formamidine insecticides (tobacco
598 budworm), paraoxon (*Pieris* butterflies), but not to essential oils (Mediterranean flour moth)
599 (reviewed in Campbell *et al.*, 2016). Fumigation has been found to be effective against the
600 Indian meal moth (*Plodia interpunctella*), a lepidopteran stored product pest (reviewed in
601 Campbell *et al.*, 2016), and it is known that butterflies appear to have a high susceptibility to
602 the transovarial transport of pyriproxyfen (Steigenga *et al.*, 2006). To date, no studies have
603 examined the susceptibility of lepidopteran eggs to entomopathogenic fungi, or examined the
604 potential for enzymatic resistance in lepidopteran embryos (Campbell *et al.*, 2016). Together,
605 these data suggest that in Lepidoptera the chorion can form a very effective mechanical
606 barrier against some, but not all pesticides. During early embryogenesis of pterygote insects,
607 such as butterflies, another barrier forms which consists of an epithelial sheet of cells called
608 the serosa that can actively express relevant genes to process environmental toxins (Berger-
609 Twelbeck *et al.*, 2003). As such, there is a huge potential for the serosa to play an active role

in protecting butterfly embryos from pesticides, but at present, no studies have examined whether this is a mechanism of particular significance for butterflies.

Many studies of insects other than butterflies have demonstrated that alteration of the molecular targets of insecticides, most commonly by mutation, is associated with resistance (reviewed in Ffrench-Constant *et al.*, 2016). For example, a point mutation in the gene encoding the γ-aminobutyric acid (GABA) receptor RDL (resistant to dieldrin) gives rise to resistance to dieldrin and several other insecticides in a variety of species including the diamondback moth *P. xylostella* (Wang *et al.*, 2016). The presence of such mutations in butterflies may indicate exposure and adaption to certain insecticides. It is also emerging that species-specific isoforms of RDL generated by alternative splicing and RNA A-to-I editing may influence sensitivity to insecticides (reviewed in Taylor-Wells and Jones, 2017). It will be of interest to investigate whether different butterfly species have such species-specific diversification in insecticide targets and whether this contributes to differential sensitivities to insecticides displayed in various species. Indeed, we found that many relevant genes in the context of pesticide targets, but also defence against pesticides, display divergence and expansion in butterflies with respect to other insects, including unique gene duplications (i.e. paralogs) and sequence divergence (Supplementary figure 1). We have demonstrated this for the *multidrug resistance* (*mdr*) genes (Supplementary figure 1). Differential gene expression levels as well as sequence variation in *mdr* genes have been shown to be the cause of population differences in the response to toxic compounds, and the development of resistance in various insects (Begin and Whitley, 2000; Dermauw and Van Leeuwen, 2014), but these genes (including paralogs) have not been studied in Lepidoptera (Simons *et al.*, 2013). Ryanodine receptors are targets for a class of insecticides known as diamides. These appear less divergent than the *mdr* genes (supplementary figure 2), illustrating divergence in evolutionary rate between gene families. Although well-studied in moths (including pesticide

635 resistance; e.g. Bird, 2016; Steinbach *et al*, 2015), no data on these receptors and the effects
636 of diamides exist for butterflies (Supplementary File). Establishing natural variation in such
637 genes (including the significance of the paralogs) and how it may underpin differences in
638 pesticide sensitivity between butterfly populations is an exciting future research area.

639

640 **6. Conclusions and future research**

641 This review highlights the need for integrated studies examining the impact of
642 pesticides on butterflies which combine data across multiple scales; from direct toxicity tests
643 on individual larvae in the laboratory to field studies that consider the potentiation of
644 pesticides by ecologically relevant environmental biotic and abiotic stressors. Such
645 integration would better inform population-level responses locally, regionally and nationally
646 (e.g. see Figure 1). There are several important areas which require further work in order to
647 fully understand the impact of pesticides on butterflies in nature. Little is known about
648 pesticide toxicity to butterflies, particularly in relation to differences in sensitivity across life
649 stages and species, and further work is required to determine the potential routes by which
650 butterflies may be exposed to pesticides in nature. Sub-lethal pesticide effects could severely
651 impact fitness, population recruitment and hence population size, but the larval effects also
652 remain largely unexplored. Sub-lethal effects of pesticides can also result in strong selection.
653 Transgenerational transfer of pesticides from mothers to offspring during oviposition adds an
654 additional temporal effect, which may play an important role in the population dynamics of
655 some species, and thus warrants further examination. For many pesticides, we have little
656 information about the range of field doses likely to be encountered by butterflies, or the
657 duration of exposure. We know that some pesticides, like neonicotinoids have half-lives in
658 soil exceeding 1000 days (Bonmatin *et al.*, 2015; Yadav *et al.*, 2015), so there is a high

potential for repeat exposure to some pesticides both within and across butterfly life stages. Yet, limited data are available on the sensitivity of butterflies to neonicotinoids within and across life stages (Wood and Goulson, 2017). Other questions that remain unanswered include; how do different land use types affect the impact of pesticides on non-target butterflies? How do pesticides other than insecticides affect butterflies? Does time influence how butterflies react to pesticides? Can butterflies learn to avoid affected areas or even evolve resistance as seen in other species (Konopka *et al.*, 2012; Wang *et al.*, 2013; Tabashnik *et al.*, 2014; Bass *et al.*, 2015; Sparks and Nauen, 2015)? Is there the potential for the negative effects of pesticides to be missed if different populations of butterflies are well connected, and thus when analysing data at the landscape level is it worthwhile considering whether species repeatedly recolonise habitat patches or whether they are closed communities? As was demonstrated for the Diamondback moth, *Plutella xylostella* (Hoang *et al.*, 2011; Arena and Sgolastra, 2014; Steinbach *et al.*, 2015; Yao *et al.*, 2016), it is known that different species, and even populations of the same species, can respond differently to exposure to pesticides. These differences probably have a genetic underpinning, and exploring the underlying genetic mechanisms might help us to better understand species responses to pesticide exposure. Furthermore, we also need to consider the impact of non-industrial use of pesticides in gardens, parks and other recreation areas such as golf courses, which are increasingly important in agricultural and urbanised landscapes (Colding and Folke, 2009).

Butterflies have a rich history of research in the field of evolutionary ecology, as well as their physiological responses to environmental variation. Recently these fields have become increasingly more integrated by investigating the underlying developmental genetic mechanisms involved in the response to a variety of environmental factors, in particular host plants (Yu *et al.*, 2016; Schweizer *et al.*, 2017; Sikkink *et al.*, 2017). Speckled Wood

butterflies (*P. aegeria*), for example, are an emerging developmental genetic model system to study growth, development (including embryogenesis) and the production of reproductive cells (Carter *et al.*, 2013; Carter *et al.*, 2015; Schmidt-Ott and Lynch, 2016). It is also a species whose habitat has expanded from forests to include agricultural fields and urbanised environments, providing an opportunity to gauge the effects on pesticide exposure on local populations in a (meta-)population network (Van Dyck and Holveck, 2016). Given the fact that many pesticides affect development, growth and reproduction (e.g. hormone analogues such as pyriproxyfen), as well as general metabolism, physiology and behaviour (e.g. neonicotinoids), it is timely to investigate the effects of pesticides on butterflies from the molecular level all the way to the population dynamic level using species such as *P. aegeria*. Research on relevant genes in moths, as well as other insect orders, in particular the Diptera (e.g. *Drosophila* and mosquitoes), provides us with a starting point to examine candidate mechanisms and genes (Feyereisen *et al.*, 2015). Having identified relevant genes involved in the pesticide response one can thus investigate which genes are likely to be under selection and involved in differential pesticide responses and resistance among populations within a species but also among species (see supplementary information). Furthermore, different life-stages may differ in their sensitivity to pesticides to differential expression levels of the relevant genes. Finally, such detailed information will allow us to make more robust predictions of the fate of individual populations under a range of environmental conditions, and how they may affect life-history evolution.

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Supplementary material

Supplementary file contains 1) a detailed overview table of research examining the effects of direct pesticide exposure on different butterfly species, and 2) phylogenetic analyses and discussion of the *multidrug resistance* (*mdr*) genes and genes encoding Ryanodine receptors

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Table 1: A summary of the butterfly species, stages and pesticides used in direct pesticide exposure studies. First column contains the species tested, second column indicates which stages in the lifecycle were tested, and the third column the pesticides used. Definitions of terms in the table; *E* refers to egg stage, *L* refers to all possible instars of larval development, *A* refers to adult stage. Supplementary table 1, summarises the main findings of each paper in more detail, including the doses used.

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Species	Stage	Pesticide	Reference(s)
Aglais urticae	L	p-p'-DDT, Dieldrin	Moriarty (1968)
Agraulis vanilla	L, A	Naled, Malathion	Eliazar and Emmel, 1991; Salvato, 2001
Anartia jatrophae	L, A	Permethrin, Naled, Dichlorvos	Hoang *et al.*, 2011; Hoang and Rand, 2015
Ascia monuste	A	Naled	Bargar, 2012a,b
Bicyclus anynana	A	Pyriproxyfen	Steiginga *et al*, 2006
Danaus plexippus	L, A	Clothianidin, Imidacloprid, Permethrin	Oberhauser *et al.*, 2006; Krischik *et al.*, 2015; Pecenka and Lundgren, 2015
Dryas julia	A	Naled	Bargar, 2012a
Eumaeus atala	L, A	Permethrin, Dichlorvos, Naled	Salvato, 2001; Hoang *et al.*, 2011; Hoang and Rand, 2015
Heliconius charitonius	L, A	Permethrin, Naled, Dichlorvos, Fenthion, Malathion	Eliazar and Emmel, 1991; Salvato, 2001; Hoang *et al.*, 2011
Icaricia icarioides blackmorei	L	Surfactant, Fluazifop-*p*-butyl, Sethoxydim	Russell and Schultz, 2010
Junonia coenia	L, A	Permethrin, Naled, Dichlorvos	Hoang *et al.*, 2011; Bargar, 2012a
Neophasia menapia	L	SBP-138, Pyrethrins, Dewco-214, Methomyl, Chlorpyrifos, Tetrachlorvinphos, Sumithion, Phoxim, Zectran, Aminocarb, Malathion, Carbaryl, DDT, Trichlorfon	Lyon and Brown, 1971
Papilio cresphontes	L, A	Naled, Fenthion, Malathion, Resmethrin	Eliazar and Emmel, 1991
Papilio demoleus	L	β-Asarone, Diofenolan	Singh and Kumar, 2011; Vattikonda *et al.*, 2015
Papillo spp	E	BHC, Dicrotophos, Chlorfenvinphos, Carbaryl, Diazinon, Dichlorovos, Dimethoate, Formothian, Malathion, Methamidophos, Parathion, Phosphamidon, Quinalphos, Tricholorofon	Siddappaji *et al.*, 1977
Pieris brassicae	E, L	Paraoxon, Deltamethrin, Dimethoate, Pirimicarb, Phosalone, Endosulfan, Fenitrothion, Pirimiphos-methyl, Fenvalerate, Diflubenzuron, Cypermethrin, Permethrin, λ-cyhalothrin, Alphametrin, Bifenthrin, β-cyfluthrin, Fenpropathrin, Fenvalerate, DE / New silica, Spinosad, Diazinon, Diazoxon, Triazophos, Dimethoate, Dichlorvos, Quinolphos, Carbaryl, Pirimicarb	David, 1959; Wahla *et al*., 1976; Tan, 1981; Sinha *et al*., 1990; Davis *et al*., 1991a; Davis *et al.*, 1993; de Jong and van der Nagel, 1994; Çilgi and Jepson, 1995; Bhat *et al*., 1997; Klokočar-Šmit *et al*., 2007; Dhingra *et al*., 2008; Mucha-Pelzer *et al*., 2010
Pieris napi	L	Dimethoate, Phosalone, Fenitrothion, Diflubenzuron	Davis *et al.*, 1991b
Pieris rapae	L	Surfactant, Fluazifop-*p*-butyl, Sethoxydim, Deltamethrin, Pumpkin leaf acetone extract	Çilgi and Jepson, 1995; Xu *et al.*, 2008; Russell and Schultz, 2010
Polymmatus icarus	L	Fenitrothion, Clothianidin	Davis *et al.*, 1991b; Basley and Goulson, 2018
Proteus urbanus	L, A	Naled, Malathion	Salvato, 2001
Pygrus oileus	L, A	Naled	Salvato, 2001
Pyronia tithonus	L	Fenitrothion, Diflubenzuron	Davis *et al.*, 1991b
Vanessa cardui	L, A	Permethrin, Naled, Dichlorvos, Fenthion, Malathion, Resmethrin, Imidacloprid	Hoang *et al.*, 2011

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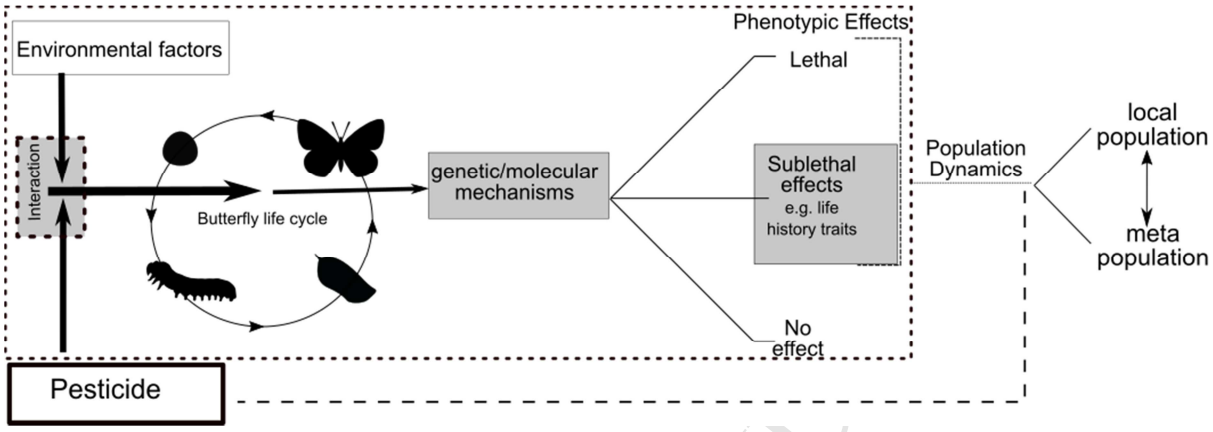

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Figure 1: The complex effects of pesticides on butterflies

The effects of pesticides on butterflies are poorly understood, the dashed area outlined in the figure highlights where future research efforts are needed. Highlighted in grey are the 3 main areas where further research is required; 1) the effects of pesticides in interaction with biotic and abiotic environmental factors at different life stages,. 2) the effects at the molecular level, particularly in non-target organisms, and determination of which genes are of importance in defence (and thus possibly resistance), and 3) how the effects of the pesticide manifest themselves at the phenotypic level (via lethal, sublethal, life history traits (e.g. reproduction) or even possibly from having no effect). Published meta-analyses have tried to infer from population dynamic trends what the pesticide effects were at the level of the individual (indicated by the broken line at the bottom of the figure joining pesticide and population dynamics).

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Highlights for the paper

The effects of insecticides on butterflies – a review

- Butterflies are often innocent targets of large-scale pesticide use, but information is scarce
- Integrated research approach across life-cycle stages, from the molecular/genetic level up
- Research needed on the interaction between environmental factors and pesticides
- Inferring effects of pesticides by studying population dynamics only may be misleading

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