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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

67 butterflies, extrapolation will be possible to those benign moth species that have seen their

68 numbers reduced, not least due to indiscriminate effects of pesticides (Fox, 2012).

69   Benefits of using pesticides in agriculture range from nutritional health and/or

70 increased diversity of viable crops, to more derived secondary benefits such as a reduced

71 migration by humans to cities and a better educated population (Cooper and Dobson, 2007;

72 Aktar *et al.*, 2009). On the other hand, the increased use of pesticides can also result in

73 harmful side-effects for wildlife (Boutin *et al.*, 1999; Bell *et al.*, 2001; Mineau, 2005). While

74 such negative impacts of modern, intensive agriculture on biodiversity have been widely

75 recognised, the contribution that agricultural pesticides make to this overall impact has

76 largely been neglected (Gibbs *et al.*, 2009; Gilburn *et al.*, 2015). Insecticides are one of the

77 biggest classes of pesticides used in the world (Aktar *et al.*, 2009), and this review reflects

78 that insecticides are also the class of pesticides predominatly investigated in butterflies.

79 Although insecticides are produced as a pest preventative method, the vast spectrum of their

80 toxicity inadvertently leads to the suppression of non-target insects and organisms inhabiting

81 the same niche or environment. Affected, non-target organisms might include pollinators,

82 natural predators and parasites (Johansen, 1977).

83   The main focus of research on non-target pesticide effects has been the European

84 honey bee (*Apis melligera)* (Sanchez-Bayo and Goka, 2014)*.* The honey bee is the most

85 economically valuable pollinator of crop monocultures and their absence could cause a

86 decrease in yield of up to 90% in some crops (Southwick and Southwick, 1992; Winfree *et*

87 *al.*, 2007; Arena and Sgolastra, 2014). In recent years many (managed) bee colonies suddenly

88 died over winter, through a phenomena named Colony Collapse Disorder (CCD)

89 (vanEngelsdorp *et al.*, 2009). The cause of CCD is unknown and is probably the result of a

90 complex interaction between multiple factors. One of the factors implicated in CCD are

91 pesticides, especially neonicotinoids (Ratnieks and Carreck, 2010; van der Sluijs *et al.*, 2013;

92   Lu *et al.*, 2014; Pisa *et al.*, 2015). Neonicotinoids are the most used class of pesticides in the

93   world. They are widely applied as seed dressing and work systemically throughout the plant.

94   Neonicotinoids mimic the acetylcholine neurotransmitter and are highly neurotoxic to insects

95   (Goulson, 2013; van der Sluijs *et al.*, 2013; Crossthwaite *et al.*, 2017). The indication of their

96   role in CCD caused the European Union to ban three pesticides in the class of neonicotinoids

97   in 2013, namely clothianidin, thiamethoxam and imidacloprid (European-Commission,

98   2013). The observation of CCD and the consequent neonicotinoid ban renewed and

99   intensified the interest and research into the (non-target) effects of neonicotinoids in

100 particular and pesticides in general (e.g. Pisa *et al.* (2015); Woodcock *et al.* (2016); Wood

101 and Goulson (2017); Woodcock *et al.* (2017))

102   Although honey bees are cheap, versatile, easy to manage and create their own

103 economically valuable product they are not the most effective pollinator for a lot of crops

104 (Klein *et al.*, 2007). Furthermore, honey bees are not the only non-target species affected. A

105 recent review by Pisa *et al.* (2015) assessing the impact of pesticides on non-target species,

106 identified a need for studies investigating the effect of pesticides on Lepidoptera, in particular

107 butterflies (see also Wood and Goulson (2017)).

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109 *1.2 Butterflies as models for non-target effects of pesticides*

110   Butterflies play an important role in ecosystems as plant pollinators (Feber *et al.*,

111 1997; Potts *et al.*, 2016) and as prey for other organisms (Strong *et al.*, 2000). Well-known to

112 the general public, they are well monitored, recognised as indicators of environmental health

113 (Whitworth *et al.*, 2018) and as such they have been used to measure impact of factors such

114 as climate change (Schweiger *et al.*, 2012) and landscape fragmentation (Scriven *et al.*,

115 2017). Comparatively, their ecology and abundance is much better known than any other

116   invertebrate taxa (New, 1997). This allows the possibility to investigate the impact of

117   pesticides across a large ecological range (Fontaine *et al.*, 2016). Butterfly species diversity

118   and abundance has already been shown to be influenced by landscape complexity and type of

119   farming (Rundlöf and Smith, 2006), quality of habitat (Pocewicz *et al.*, 2009) and habitat

120   management (Marini *et al.*, 2009). Obviously some butterfly species are agricultural pests,

121   such as the cabbage white species (*Pieris* sp.), but nothing like the scale and species diversity

122   observed for moths (Feber *et al.*, 1997). Understanding butterflies' sensitivity and responses

123   to pesticide exposure more fully might help assess the overall risk of pesticide use (Pisa *et al.*,

124   2015). The availability of genomic data for an ever-increasing number of butterfly species

125   allows one to investigate the observed sensitivity and responses at the underlying molecular

126   level (Shen *et al.*, 2016; Liu *et al.*, 2018), but also how they may adapt to agricultural

127   environments (Sikkink *et al.*, 2017). Research at the level of such integration in butterflies is

128   far behind that of moths, and thus the detailed studies on pesticide development, usage and

129   effects on pest moths can provide valuable starting points for such an approach (Troczka *et*

130   *al.*, 2017)

131   The habitat of many butterfly species consists of hedgerows or the fragmented areas

132   between arable lands (Warren *et al.*, 2001; Krauss *et al.*, 2003). Butterflies can therefore

133   come into contact with pesticide treated plants and areas through foraging or translocation.

134   Butterflies inhabiting hedgerows are susceptible to spray drift from insecticides (Davis *et al.*,

135   1991a; 1991b; Çilgi and Jepson, 1995; Kjær *et al.*, 2014). Numbers of widespread butterflies

136   on monitored farm land have declined by 58% between 2000 and 2009 (Brereton *et al.*,

137   2011), and a number of species are under threat. Some pesticides are applied in the form of a

138   coating around seeds, this coating leaves a residue in the soil, and if water-soluble this

139   residue can enter the ground water (Bonmatin *et al.*, 2015; Schaafsma *et al.*, 2015). Uptake

140   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.

190   Pekin (2013) used a large scale dataset, not focusing on absolute abundance of

191   butterflies in the analyses, but rather on the number of butterfly species. This study found that

192   variation in Turkish butterfly species composition was largely explained by the combination

193   of agricultural chemical use, especially pesticides, with climate and land-cover variables. The

194   significance of these variables varied per Turkish province, and thus location. Muratet and

195   Fontaine (2015) used a large-scale dataset, collected by the public which considered pesticide

196   use and butterfly abundance in their gardens. Pesticides, especially insecticides and

197   herbicides were found to have a negative impact on butterfly abundance. This study

198   examined an aspect of pesticide use often overlooked; the non-industrial use of pesticides.

199   Although these effects might be smaller, gardens can be very important refuges for butterflies

200   (Fontaine *et al.*, 2016).

201   The other four studies compared sets of similar land types where the biggest

202   difference across treatments was the amount of pesticide used. Feber *et al.* (2007) and Feber

203   *et al.* (1997) used paired sets of neighbouring organic and non-organic farms to compare

204   butterfly abundance. Both of these studies found that irrespective of the type of crop present,

205   non-pest butterfly species were more abundant on organic farms, especially in the uncropped

206   field margins. Brittain *et al.* (2010) used a pair of intensively farmed basins in Italy versus a

207   nature reserve and compared whether intensively farmed land with high pesticide use had

208   lower species richness than the nature reserve, which had negligible amounts of pesticide use.

209   This study found that at the regional scale, butterfly species richness was lower in the

210   intensely farmed basin with the high pesticide loads. Salvato (2001) surveyed 9 transects in

211   South Florida and Lower Florida Keys for adult and larval densities of three species of

212   butterflies; *Anaea troglodyte, Strymon acis bartrami* and *Hersperia meskei*. All pesticide

213   treatment areas were compared against controls; areas where insecticide applications are

214   restricted. In most cases, there was a lower butterfly density in the sprayed locations

215   compared to the control sites. Larval density seems to be highest in unsprayed transects, and

216   increased in transects that ceased insecticide application.

217   Finally, as mentioned previously, the studies of Gilburn *et al.* (2015) and Forister *et*

218   *al.* (2016) differ from the other six studies in the approach they used to study the impact of

219   pesticides on butterfly abundance. Gilburn *et al.* (2015) used UK-wide abundance data of 17

220   widespread resident butterfly species that routinely breed in any field or field margin habitats

221   for their analysis. They modelled data from 1985 to 2012 and their model included a whole

222   range of current and previous year weather measurements such as mean temperature and

223   rainfall during the seasons, as well as the previous year's population index for each species

224   and previous year's pesticide use. A strong negative correlation between butterfly population

225   size and the amount of neonicotinoids used in previous years was observed. In 1998

226   neonicotinoid use in the UK exceeded 100,000 hectares for the first time. To examine the

227   impact of this increase in neonicotinoid usage on butterfly abundance, Gilburn et al. (2015)

228   split their data set up into two different time periods, one from 1985 to 1998 and one from

229   1998 to 2012. Remarkably, when the same model was applied to analyse variation in

230   butterfly abundance across these two-time periods, the abundance of widespread butterflies

231   showed a significant increase in the first -1985 to 1998- dataset, and a decrease in the second

232   -1998 to 2012. These data suggest that increased usage of neonicotinoid pesticides may

233   correlate with a decline in the abundance of 17 widespread UK butterfly species.

234   Forister *et al.* (2016) used a somewhat similar approach to the Gilburn *et al.* (2015)

235   study but over a smaller geographical scale using longitudinal data from 4 North Californian

236   locations experiencing butterfly declines since the late 1990's . In each of the locations the

237   presence of 67 butterfly species was monitored on a bi-weekly basis for 40 years. A negative

238   relationship between neonicotinoid use and annual variation in butterfly species observations

239   was readily detectable, while controlling for land use and other factors. Furthermore, smaller-

240 bodied butterfly species and those with fewer generations per annum showed more severe

241 declines in response to neonicotinoid exposure.

242   Even though these eight studies used a wide variety of different experimental and

243 statistical approaches to examine the response of butterfly species over a range of spatial and

244 temporal scales, a similar trend was reported by all; increased pesticide levels lead to

245 reductions in butterfly abundance or species richness. The trends reported in these articles are

246 in line with general expectations i.e. pesticide use can have detrimental non-target effects on

247 butterflies. However, these studies do highlight some other important and interesting factors

248 that require further consideration. One of these is consideration of how much non-industrial

249 use of pesticides might affect vulnerable species, especially in places like gardens which are

250 increasingly being used in urbanised landscapes by many butterfly species as habitat patches

251 that provide essential resources such as nectar sources and host plants for oviposition

252 (Fontaine *et al.*, 2016). More detailed research into this area would be very valuable (Muratet

253 and Fontaine, 2015), especially because butterfly abundance and species richness have been

254 shown to be negatively correlated with pesticide use in gardens (Fontaine *et al.*, 2016).

255   Studies examining population trends to determine the non-target effects of pesticides

256 on butterflies are very informative as the effects of pesticides are complex, and looking at the

257 real-world effects can give vital insight into the actual scale of the effect. These studies also

258 provide an opportunity to explore the impact of indirect effects, for example through complex

259 interaction and by reducing the number of suitable host plants. Although factors, such as

260 weather, interacting with pesticide use should be taken into account, this is not always done,

261 through a lack of power in the dataset. A vast number of butterfly species utilise host plants

262 commonly considered to be weeds, which may be targeted by herbicides (Malcolm, 2018).

263 Whilst crops may be genetically modified to develop herbicide resistance, other plants may

264 be affected by herbicide spray drifts. This reduction in host plant availability or quality may

265   also lead to reduction in butterfly abundance without having any direct toxicity effects on

266   butterflies (Smart *et al.*, 2000). In Feber *et al.* (2007) this idea was explored by comparing

267   differences in botanical compositions between the organic farms and conventional farms.

268   Although no difference in grass and forb species between organic and conventional field

269   boundaries was found, there may be differences in the abundances of particular nectar

270   sources and host plants, which could impact butterfly population dynamics.

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272   *3.2 Field studies*

273   Studies addressing the effects pesticides on butterflies, as well as genes involved, in a

274   field context are based on butterflies that are considered pest species, including *Pieris*

275   *brassicae* (cabbage butterfly), *Pieris rapae* (small cabbage white butterfly)*, Pieris napi*

276   (green-veined white), *Virochola livia* (pomegranate butterfly)*, and Papilio demoleus* (lemon

277   butterfly) (Liu *et al.*, 2018). Such studies do not examine effects on non-target butterfly

278   species. However, they do give a good insight into the actual field efficacy and thus the

279   potential level of harmfulness to butterflies in general, particularly because the method of

280   application, as well types of areas where some pesticides are applied suggest the potential for

281   affecting non-target butterflies.

282   First, we will discuss studies focussing on *P. brassicae* as a target species. Davis *et al.*

283   (1991b) compared the pesticide sensitivity of larvae from three butterfly species in the lab

284   and established that *P. brassicae* as tested by Sinha *et al.* (1990) showed higher sensitivity to

285   the following tested insecticides; Dimethoate, Phosalone, Fenitrothion and Diflubenzeron.

286   This led them to conclude that *P. brassicae* might be a good indicator species for the effects

287   of pesticides on butterflies in general (Davis *et al.*, 1991b). Subsequently both *P. brassicae*

288   and *P. napi* larvae were exposed to the same spray drift at field-realistic concentrations,

289 which again showed *P. brassicae* to be the more sensitive species to the pesticide

290 diflubenzuron, another insecticide. The molecular mechanisms or other reasons why *P.*

291 *brassicae* seems to be more sensitive to pesticides than the other tested species were not

292 addressed. Muthukumar *et al.* (2007) and Thakur and Deka (1997) combined, tested 19

293 different pesticides for their efficacy to kill or deter *P. brassicae* larvae. All of these 19

294 treatments had a significant effect, greatly reducing the number of larvae. Thakur and Deka

295 (1997) mention six pesticides (deltamethrin, cypermethrin, malathion, fenitrothion,

296 endosulphan and monocrotophos) with a field efficacy higher than 90%, and one, fenvalerate,

297 had a field efficacy of 100%. These numbers indicate that these pesticides are highly toxic to

298 *P. brassicae*, and potentially toxic to other butterfly species too. As these pesticides are

299 applied by spray there is a high possibility of drift and thus contact with non-target

300 butterflies.

301   Another frequently investigated pest species is the pomegranate butterfly (*V. livia*), in

302 countries including Egypt, Cyprus and Jordan (Obeidat and Akkawi, 2002; Kahramanoglu

303 and Usanmaz, 2013; Abd-Ella, 2015). *Virachola livia* lay their eggs on fruit, and after

304 hatching the larvae bore into the fruit, causing crop damage. In contrast to the aforementioned

305 *P. brassicae* studies, larval mortality levels were not measured. Instead, the reduction of fruit

306 infestation and fruit damage after pesticide application was studied. Although a reduction in

307 fruit damage was observed, the mechanism underlying this reduction is unknown, and it is

308 unclear whether it is due to pesticides acting as an oviposition deterrent, or due to the

309 pesticides directly killing eggs or larvae. A closer look into the mechanisms of crop

310 protection could help to indicate the possible non-target toxicity effects on other butterflies

311 and insects. These studies indicate that a wide range of pesticides may have high field

312 toxicity to butterflies, suggesting that numerous, different pesticides are highly likely to have

313 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

was used and in what dose was not reported. The four types of headlands also had

significantly fewer butterflies in the field areas with fully sprayed headlands. Furthermore,

the pierids *Anthocharis cardarnines, P. napi* and *P. rapae* all managed to lay eggs in the

conservation headland on their host plants *Sinapis arvensis L.* and *Brassica napus*, be it in

low densities. A similar study conducted in the Netherlands also reported fewer butterflies in

sprayed margins than in unsprayed margins. It did depend both on the crop type and the year

examined (Snoo *et al.*, 1998). It can be hypothesised that the favourable effects on butterfly

abundance in the unsprayed margins were mainly due to the greater availability of flowering

plants but could not be tested with the data from Snoo et al (1998). Such hedgerow studies

also provide some insights not only into indirect effects of pesticides but also into potential

interaction effects with other factors. An example includes the effects of herbicides and

fertilisers on butterflies and their associated hostplants (Longley and Sotherton, 1997).

Spray drift is named as one of the main sources of non-target butterfly exposure to

pesticides, as pesticides drift over from fields of arable land to areas with higher number of

resources for butterflies such as hedgerows, wildflower patches or even nearby nature

reserves (Sinha *et al.*, 1990; Zhong *et al.*, 2010). Quite a few studies examine ground-level

spraying effects on butterflies (Davis *et al.*, 1991a, b, 1993, 1994; de Jong and van der Nagel,

1994), while Zhong *et al.* (2010) addressed the impacts of aerial ultra-low volume spraying of

Naled on the Miami blue butterfly in Florida. Naled is used to target mosquitoes and a small

droplet of Naled created by the ultra-low volume spraying does not settle quickly and is

capable of drifting extended distances both in and out of the target area. The Miami blue

butterfly (*Cyclargus thomasi bethunebakeri*) is endemic to Florida and has been in serious

decline. In addition to habitat loss, climate change and a handful of other factors, the use of

the aerial application of Naled has been indicated as a possible contributory factor in their

decline. Naled was found to negatively affect late instar Miami blue larvae at the

389   concentration found in the target zone, but not at the concentrations found in the spray drift

390   zones (Zhong *et al.*, 2010). However, whether the concentrations of Naled found in the spray

391   drift zones affects other larval instars or life stages of these butterflies requires further work

392   (Zhong *et al.*, 2010).

393   However, it was found that even at low wind levels pesticides could drift and cause

394   high mortality to *P. brassicae* larvae up to 24 metres away from the spray site (studies

395   reported in table 1 and Supplementary File). For example, Davis *et al.* (1994) monitored 2-

396   day-old *P. brassicae* were placed on plants at different distances from a field sprayed with

397   cypermethrin, recording a higher mortality of larvae for three days after spraying. They

398   included an examination of how landscape features, especially hedgerows, could influence

399   the spread of pesticides by spray drift, by acting as a barrier, and concluded that hedges may

400   provide a sheltered area immediately behind the hedge, but as the distance from the hedge

401   increases, larval mortality increases again minimising the shelter effect of the hedge. de Jong

402   and van der Nagel (1994) also placed *P. brassicae* at different distances from a plot of land

403   sprayed with diflubenzuron. In this study the LD-50 was established at only 0.16% of the

404   sprayed dose, and the drift from the application was at a sufficiently high concentration to

405   still cause larval mortality. As expected, the closer the larvae were to the sprayed area the

406   higher were the mortality levels. These studies indicate that pesticide spray drift has the

407   potential to cause serious mortality in butterfly species over considerable distances from the

408   sprayed area, and that landscape features, such as hedges, are ineffective barriers to spray

409   drift.

410

411   *3.3 Direct toxicity effects of pesticides on butterflies*

412   Here, we were interested in determining how many different butterfly species have

413   been used in direct toxicity tests, which pesticides have been tested on butterflies, in what

414   dose and which butterfly life stages have been examined. For example, recent studies on *P.*

415   *rapae* dissecting the sensitivity and response to pesticides at the molecular level (e.g.

416   identification of relevant genes) do so in a life-stage specific way (Liu *et al.*, 2017; Liu *et al.*,

417   2018).

418   In total, 22 species of butterflies were used in direct toxicity tests of pesticides (Table

419   1). It should be noted that these were all insecticides. Ten of these species were exposed to

420   such pesticides in both the larval and adult stages and one species, *P. brassicae,* was used in

421   egg and larval stage. Three species, *Ascia monusta, Bicyclus anynana and Dryas julia*, were

422   only tested in the adult stage and the remaining eight species were only tested in the larval

423   stage. The number of studies published per species is highly variable, ranging from a single

424   study for the majority of species studied, to 12 different studies on *P. brassicae.* As

425   mentioned earlier in this review, *P. brassicae* has been demonstrated to be more sensitive to

426   pesticides than some of the other species studied, and has therefore been suggested to be a

427   good model species for examining the impact of pesticides on butterfly pest species (Davis *et*

428   *al.*, 1991b). This may explain why the majority of studies examining effects of pesticides are

429   on this species. In total, we found 31 studies that examined the direct effects of pesticide

430   exposure on butterflies (Table 1). The majority of these studies performed direct toxicity tests

431   on the larval stage (n= 26 studies), a few have considered the adult stage (n = 8 studies), but

432   hardly any studies have examined the impact of pesticide usage in the egg stage (n = 2) and

433   none examined the pupal stages in butterflies (Table 1). Few studies have considered the sub-

434   lethal effects of pesticides through the different stages of the life cycle to the adult stage, or

435   considered potential for transgenerational effects (i.e. the transfer of the effects of pesticides

436   from parents to offspring). Although the larval stage is probably the most economically

437 damaging phase of the butterfly life cycle, and thus the most suitable part of the life cycle to

438 target for pest control, it would be valuable to examine how pesticides impact other life

439 stages to provide further insights into the non-target and sub-lethal effects of pesticides on

440 butterfly populations.

441   In the studies detailed in Supplementary table 1, butterflies have been directly

442 exposed to pesticides (i.e. insecticides) using 3 main methods; 1) direct physical exposure,

443 bringing a droplet of pesticide of a specific concentration straight on to, often the thorax, of

444 the larvae or adult butterfly, 2) using a similar method to 1 in which the egg, caterpillar or

445 adult butterfly was sprayed with, or otherwise physically exposed, to a pesticide and 3) larvae

446 are exposed to food plants treated with a pesticide. Additionally, in two studies the larvae

447 were exposed via a plant grown on pesticide treated soil (Krischik *et al.*, 2015; Basley and

448 Goulson, 2018).

449   A wide range of pesticides have been tested for their toxic effects on butterflies, and

450 19 of these studies report a LD-50 for that pesticide under their tested conditions

451 (Supplementary table 1). Although these values give a rough indication of the toxicity of each

452 particular pesticide for butterflies, there are a number of factors that may affect the generality

453 of these findings. First, the response to any given pesticide is likely to be very species-

454 specific. The study by Hoang *et al.* (2011) provides a good example of why it is important to

455 consider species-specific responses to pesticides. They exposed 5th instar larvae of four

456 different butterfly species to the pesticide Naled. The range of LD-50 at 24 hours after

457 exposure lies between 0.19 μg/g for *Anartia jatrophae* and 10.82 μg/g for *Vanessa cardui*,

458 which means that a fifth instar *A. jatrophe* caterpillar is almost 57 times more sensitive to

459 Naled than a fifth instar *V. cardui* caterpillar. This is a difference that cannot solely be

460 explained by a difference in larval size as *V. cardui* 5th instar larvae (0.553±0.05 g) are only

461 1.3 times heavier than *A. jatrophe* 5th instar larvae (0.425±0.012 g).

462   Second, the response to a pesticide is highly dependent on the life stage of the

463   butterfly examined; a first instar caterpillar might be more sensitive than the fourth instar

464   caterpillar of the same species (reviewed in Wood and Goulson (2017)). This effect is well

465   demonstrated by the results of Eliazar and Emmel (1991), showing that different stages of the

466   life cycle have different levels of sensitivity to pesticides and that these patterns are not

467   predictable and depend on the pesticide examined. Fourth instar larvae of *Papilio cresphontes*

468   have an LD-50 of 193.01 μg/g for Fenthion and an LD-50 of 62.463μg/g for Malathion whilst

469   fifth instar larvae of the same species have LD-50s of 41.1 μg/g and 128.455 μg/g

470   respectively. For both pesticides, the sensitivity of *P. cresphontes* depended on the instar of

471   the larva but for Fenthion the sensitivity decreased, while for Malathion it increased with

472   larval age. Additionally, Davis *et al.* (1993) shows that even a couple of days can have a big

473   difference on the sensitivity of larvae to pesticides. Two-day old *P. brassicae* larvae have an

474   LD-50 of 1.521 μg/g when Triazophos is topically applied, while four-day old larvae have an

475   LD-50 of 3.283 μg/g. In the moth *Spodoptera frugiperda*, increased tolerance to the

476   pesticides methomul, diazinon and permethrin with larval age was associated with increased

477   midgut aldrin epoxidase and gluthathione S-transferase activity (Yu *et al.*, 2015). However,

478   more studies would be required to determine whether similar mechanisms are responsible for

479   the age-specific variation in insecticide susceptibility observed in butterfly larvae. The

480   mechanisms underlying these subtle changes in sensitivity and differences in trends between

481   pesticides require further investigation. This could provide valuable insights into the modes

482   of action of pesticides and determine when and how pesticides are most effective.

483   Lastly, the method of application could potentially have a large influence on the effect

484   of pesticides. Dhingra *et al.* (2008) exposed third instar of *P. brassicae* to cypermethrin in

485   two different ways; spraying the larvae with pesticide versus feeding the larvae with leaves

486   dipped in the cypermethrin. The larvae had an LD-50 of 9.0 μg/ml when fed with leaves

487 dipped in cypermethrin, versus an LD-50 of 11.6 µg/ml LD-50 when they were directly

488 sprayed. Such differences in sensitivity could have major effects in the field.

489   In order to test what effects pesticides may have, field-realistic doses should be used

490 as was done when testing the effects of the neonicotinoid clothianidin on the development

491 and survival of *Polyommatus icarus* (see Supplementary table 1; Basley and Goulson

492 (2018)). Reduced larval growth and elevated mortality levels were detected, but ideally the

493 interaction between pesticide use and other factors (e.g. climatic variables and host plant

494 quality) should be studied to get a more realistic indication of the potential effect of

495 pesticides in the environment on multiple aspects of the butterfly development.

496   In conclusion, based on the values found in these studies alone it is difficult to

497 estimate on the harmfulness of a specific pesticide to non-target butterflies, because the

498 effects of the pesticide are likely to be influenced by the environmental context and the

499 method of application used. To estimate the actual field harmfulness, we would need much

500 more detailed knowledge about normal field doses the butterflies are exposed to, at what

501 stages butterflies are most likely to be exposed, for how long or how often they will be

502 exposed and what is the most likely exposure method that will be used. Additionally, looking

503 only at lethal doses prevents the investigation of other negative sub-lethal effects of

504 pesticides which could impact fitness-related traits and butterfly abundance at the population

505 level. Sub-lethal effects of pesticides on beneficial arthropods have been found to include

506 effects on neurophysiology, larval development, moulting, adult longevity, immunology,

507 fecundity, sex ratio, mobility, navigation and orientation, feeding behaviour, oviposition

508 behaviour and learning (Desneux *et al.*, 2007; Belzunces *et al.*, 2012; de França *et al.*, 2017).

509 The compounding effect of these factors might have a negative impact on butterfly

510 abundance even if the initial pesticide exposure is not lethal. Of the 31 studies detailed in

511 Supplementary table 1, only 12 measured the sub-lethal impacts of pesticides on butterflies.

512   11 used larval traits (e.g. larval size, development time etc.), and 3 used adult traits (e.g.

513   longevity, fecundity etc.) as a measure of sub-lethal effects. A very small number (n=4)

514   measured behavioural traits, namely feeding adverse behaviour (Tan, 1981; Xu *et al.*, 2008;

515   Vattikonda *et al.*, 2015) or egg laying choice (Oberhauser *et al.*, 2006). None of the studies to

516   date have examined sub-lethal effects of pesticides on neurophysiology or immunology in

517   butterflies. Consideration of whole-organism sub-lethal effects would be very valuable to

518   provide more realistic estimates of the longer-term impact of pesticides on butterfly

519   abundance. Synergistic effects may also play an important role in nature. Synergy occurs

520   when the effect of a combination of stressors is higher than the sum of the effect of each

521   stressor alone (van der Sluijs *et al.*, 2013). The impacts of immunity on moths are already

522   known for three pesticide classes; botanical insecticides, inorganic insecticides and insect

523   growth regulators (James and Xu (2012) provide an extensive review of mechanisms by

524   which pesticides affect insect immunity). Synergy for pesticides and pathogen infection

525   therefore has a high potential in butterflies and requires further investigation.

526

527   **4. Defence mechanisms against pesticide exposure**

528   As mentioned in the previous section, there is some evidence for differences in

529   sensitivity to pesticides both within and across life stages. We will discuss the possible ways

530   that butterflies may be able to defend themselves against exposure to pesticides across life

531   stages.

532   There are numerous different classes of pesticides specifically designed to disrupt one

533   or more different processes to cause insect mortality such as; the nervous system (e.g.

534   organophosphates, carbonates, pyrethroids, avermectins, neonicotinoids), energy production

535   (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

610   in protecting butterfly embryos from pesticides, but at present, no studies have examined

611   whether this is a mechanism of particular significance for butterflies.

612   Many studies of insects other than butterflies have demonstrated that alteration of the

613   molecular targets of insecticides, most commonly by mutation, is associated with resistance

614   (reviewed in Ffrench-Constant *et al.*, 2016). For example, a point mutation in the gene

615   encoding the γ-aminobutyric acid (GABA) receptor RDL (resistant to dieldrin) gives rise to

616   resistance to dieldrin and several other insecticides in a variety of species including the

617   diamondback moth *P. xylostella* (Wang *et al.*, 2016). The presence of such mutations in

618   butterflies may indicate exposure and adaption to certain insecticides. It is also emerging that

619   species-specific isoforms of RDL generated by alternative splicing and RNA A-to-I editing

620   may influence sensitivity to insecticides (reviewed in Taylor-Wells and Jones, 2017). It will

621   be of interest to investigate whether different butterfly species have such species-specific

622   diversification in insecticide targets and whether this contributes to differential sensitivities to

623   insecticides displayed in various species. Indeed, we found that many relevant genes in the

624   context of pesticide targets, but also defence against pesticides, display divergence and

625   expansion in butterflies with respect to other insects, including unique gene duplications (i.e.

626   paralogs) and sequence divergence (Supplementary figure 1). We have demonstrated this for

627   the *multidrug resistance* (*mdr*) genes (Supplementary figure 1). Differential gene expression

628   levels as well as sequence variation in *mdr* genes have been shown to be the cause of

629   population differences in the response to toxic compounds, and the development of resistance

630   in various insects (Begin and Whitley, 2000; Dermauw and Van Leeuwen, 2014), but these

631   genes (including paralogs) have not been studied in Lepidoptera (Simons *et al*., 2013).

632   Ryanodine receptors are targets for a class of insecticides known as diamides. These appear

633   less divergent than the *mdr* genes (supplementary figure 2), illustrating divergence in

634   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

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

659 potential for repeat exposure to some pesticides both within and across butterfly life stages.

660 Yet, limited data are available on the sensitivity of butterflies to neonicotinoids within and

661 across life stages (Wood and Goulson, 2017). Other questions that remain unanswered

662 include; how do different land use types affect the impact of pesticides on non-target

663 butterflies? How do pesticides other than insecticides affect butterflies? Does time influence

664 how butterflies react to pesticides? Can butterflies learn to avoid affected areas or even

665 evolve resistance as seen in other species (Konopka *et al.*, 2012; Wang *et al.*, 2013;

666 Tabashnik *et al.*, 2014; Bass *et al.*, 2015; Sparks and Nauen, 2015)? Is there the potential for

667 the negative effects of pesticides to be missed if different populations of butterflies are well

668 connected, and thus when analysing data at the landscape level is it worthwhile considering

669 whether species repeatedly recolonise habitat patches or whether they are closed

670 communities? As was demonstrated for the Diamondback moth, *Plutella xylostella* (Hoang *et*

671 *al.*, 2011; Arena and Sgolastra, 2014; Steinbach *et al.*, 2015; Yao *et al.*, 2016), it is known

672 that different species, and even populations of the same species, can respond differently to

673 exposure to pesticides. These differences probably have a genetic underpinning, and

674 exploring the underlying genetic mechanisms might help us to better understand species

675 responses to pesticide exposure. Furthermore, we also need to consider the impact of non-

676 industrial use of pesticides in gardens, parks and other recreation areas such as golf courses,

677 which are increasingly important in agricultural and urbanised landscapes (Colding and

678 Folke, 2009).

679   Butterflies have a rich history of research in the field of evolutionary ecology, as well

680 as their physiological responses to environmental variation. Recently these fields have

681 become increasingly more integrated by investigating the underlying developmental genetic

682 mechanisms involved in the response to a variety of environmental factors, in particular host

683 plants (Yu *et al.*, 2016; Schweizer *et al.*, 2017; Sikkink *et al.*, 2017). Speckled Wood

684   butterflies (*P. aegeria*), for example, are an emerging developmental genetic model system to

685   study growth, development (including embryogenesis) and the production of reproductive

686   cells (Carter *et al.*, 2013; Carter *et al.*, 2015; Schmidt-Ott and Lynch, 2016). It is also a

687   species whose habitat has expanded from forests to include agricultural fields and urbanised

688   environments, providing an opportunity to gauge the effects on pesticide exposure on local

689   populations in a (meta-)population network (Van Dyck and Holveck, 2016). Given the fact

690   that many pesticides affect development, growth and reproduction (e.g. hormone analogues

691   such as pyriproxyfen), as well as general metabolism, physiology and behaviour (e.g.

692   neonicotinoids), it is timely to investigate the effects of pesticides on butterflies from the

693   molecular level all the way to the population dynamic level using species such as *P. aegeria*.

694   Research on relevant genes in moths, as well as other insect orders, in particular the Diptera

695   (e.g. *Drosophila* and mosquitoes), provides us with a starting point to examine candidate

696   mechanisms and genes (Feyereisen *et al.*, 2015). Having identified relevant genes involved in

697   the pesticide response one can thus investigate which genes are likely to be under selection

698   and involved in differential pesticide responses and resistance among populations within a

699   species but also among species (see supplementary information). Furthermore, different life-

700   stages may differ in their sensitivity to pesticides to differential expression levels of the

701   relevant genes. Finally, such detailed information will allow us to make more robust

702   predictions of the fate of individual populations under a range of environmental conditions,

703   and how they may affect life-history evolution.

708

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

713

714

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1125 **Table 1: A summary of the butterfly species, stages and pesticides used in direct**

1126 **pesticide exposure studies.** First column contains the species tested, second column

1127 indicates which stages in the lifecycle were tested, and the third column the pesticides used.

1128 Definitions of terms in the table; *E* refers to egg stage, *L* refers to all possible instars of larval

1129 development, *A* refers to adult stage. Supplementary table 1, summarises the main findings of

1130 each paper in more detail, including the doses used.

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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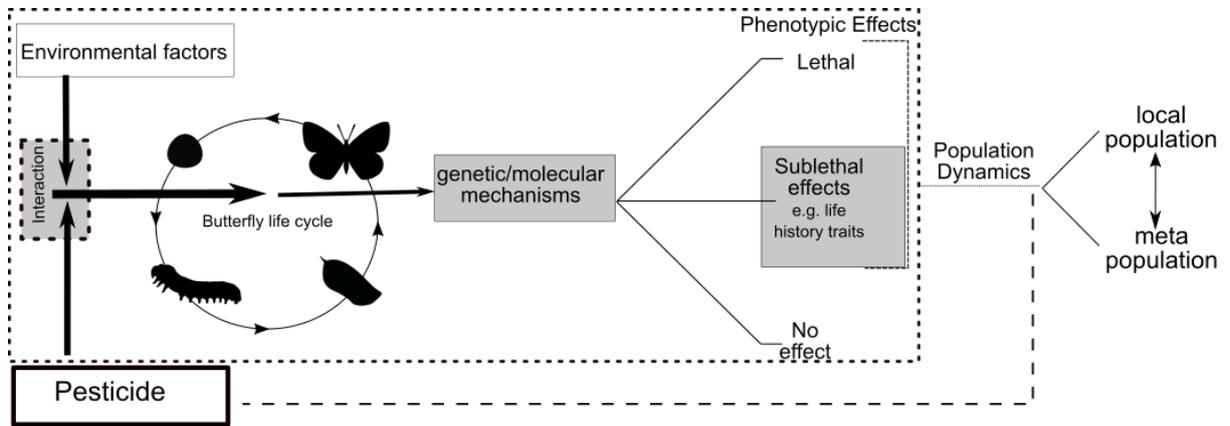
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Figure 1: The complex effects of pesticides on butterflies

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).

Supplementary table 1: An overview of research examining the effects of direct pesticide exposure on different butterfly species. The table displays the species on which direct effects of pesticides have been tested as well as the stage in their life cycle used, the pesticide tested, the method of application and main findings. The first column displays the butterfly species tested, the second column indicates at which stage in the lifecycle the species was exposed to the pesticide named in the third column, the method of pesticide application is described in the fourth column of the table. The fifth column describes the main findings in relation to pesticide toxicity found in the study named in the sixth column. Definitions of terms in the table; *Egg* refers to egg stage, *Larval* refers to all stages of larval development, *Adult* refers to adult butterflies, *LD-50* is lethal dose for 50%, *LD-90* is lethal dose for 90%, *LC-50* is lethal concentration for 50%, *AI* is active ingredient. References in the table :[1] Moriarty (1968), [2] Eliazar and Emmel (1991),[3] Salvato (2001),[4] Hoang *et al.* (2011),[5] Hoang and Rand (2015), [6] Bargar (2012b),[7] Bargar (2012a),[8] Steigenga *et al.* (2006),[9] Pecenka and Lundgren (2015), [10] Krischik *et al.* (2015), [11] Oberhauser *et al.* (2006),[12] Russell and Schultz (2010), [13] Lyon and Brown (1971) ,[14] Vattikonda *et al.* (2015), [15] Singh and Kumar (2011), [16] Siddappaji *et al.* (1977),[17] David (1959), [18] Çilgi and Jepson (1995),[19] Sinha *et al.* (1990),[20] Tan (1981),[21] Davis *et al.* (1991a),[22] Dhingra *et al.* (2008),[23] Mucha-Pelzer *et al.* (2010),[24] Klokočar-Šmit *et al.* (2007)[25] Wahla *et al.* (1976),[26] Davis *et al.* (1993),[27] Bhat *et al.* (1997),[28] de Jong and van der Nagel (1994), [29] Davis *et al.* (1991b) , [30] Xu *et al.* (2008) and [31] Basley and Goulson (2018)

Species	Stage	Pesticide	Method of application	Main findings
Aglais urticae [1]	Larval	p-p'-DDT	1 µl on the mesonotum	List of LD-50's based on weight (mg), no effect on adult longevity, fecundity or fertility
Aglais urticae [1]	Larval	Dieldrin	1 µl on the mesonotum	List of LD-50's based on weight (mg), low level adult deformation, less and infertile eggs
Agraulis vanilla [2]	Larval	Naled	1 µl onto the dorsum of the thorax	0.717 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Agraulis vanilla [3]	Larval	Malathion	1 µl onto the dorsum of the thorax	6.572 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Agraulis vanilla [3]	Adult	Malathion	1 µl onto the dorsum of the thorax	8.515 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Anartia jatrophae [4]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.79 µg/g 24h LD-50
Anartia jatrophae [4]	Larval	Naled	1 µl onto the dorsum of the thorax	0.19 µg/g 24h LD-50
Anartia jatrophae [4]	Larval	Dichlorvos	1 µl onto the dorsum of the thorax	1.13 µg/g 24h LD-50
Anartia jatrophae [5]	Larval	Permethrin	Fed on leaves dipped in insecticide solutions	1.802 µg/g 24h LD-50
Anartia jatrophae [5]	Larval	Naled	Fed on leaves dipped in insecticide solutions	0.617 µg/g 24h LD-50
Anartia jatrophae [5]	Larval	Dichlorvos	Fed on leaves dipped in insecticide solutions	1.959 µg/g 24h LD-50
Anartia jatrophae [4]	Adult	Permethrin	0.5 µl on each forewing	2.55 µg/g 24h LD-50
Anartia jatrophae [4]	Adult	Naled	0.5 µl on each forewing	1.58 µg/g 24h LD-50

Species	Stage	Pesticide	Exposure method	Result
Anartia jatrophae [4]	Adult	Dichlorvos	0.5 µl on each forewing	2.77 µg/g 24h LD-50
Anartia jatrophae [4]	Adult	Permethrin	1 µl onto the dorsum of the thorax	0.74 µg/g 24h LD-50
Anartia jatrophae [4]	Adult	Naled	1 µl onto the dorsum of the thorax	14.68 µg/g 24h LD-50
Anartia jatrophae [4]	Adult	Dichlorvos	1 µl onto the dorsum of the thorax	1.48 µg/g 24h LD-50
Ascia monuste [6]	Adult	Naled	Thorax exposure	2.0 µg/g 24h LD-50
Ascia monuste [7]	Adult	Naled	5 µl dose dorsal side of the thorax	2.4 µg/g 24h LD-50 and total cholinesterase activity is measured
Bicyclus anynana [8]	Adult	Pyriproxyfen	Between 1-100 µg in 3µl hexane topically on abdomen	Pyriproxyfen affects life-time fecundity, egg laying rate and longevity
Danaus plexippus [9]	Larval	Clothianidin	Fed on leave disk with 10µl of test substance	LC-10=7.72, LC-20=9.89, LC-50=15.63, and LC-90=30.70 ppb. Influences development time, body length, weight and head capsule size.
Danaus plexippus [10]	Larval	Imidacloprid	Fed on plant grown on soil exposed to pesticides, 300 AI mg/pot and 600 AI mg/pot	Low survival after 7 days
Danaus plexippus [11]	Larval	Permethrin	Fed on field collected leaves	Lower survival even 21 days after spraying
Danaus plexippus [11]	Larval	Permethrin	Fed on in lab sprayed plants, 0.5 and 0.1 % of operational dose (0.109 kg/ha AI)	Lower survival and longer development times
Danaus plexippus [10]	Adult	Imidacloprid	Force fed with honey and natural through flowers	No reduction in fecundity or fertility in either condition
Danaus plexippus [11]	Adult	Permethrin	Females in cages with sprayed plants	Fewer eggs laid around sprayed plants, and lower survival
Dryas julia [7]	Adult	Naled	5 µl dose dorsal side of the thorax	7.6 µg/g 24h LD-50
Eumaeus atala [4]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.08 µg/g 24h LD-50
Eumaeus atala [4]	Larval	Dichlorvos	1 µl onto the dorsum of the thorax	1.63 µg/g 24h LD-50
Eumaeus atala [5]	Larval	Permethrin	Oral /feeding	0.745 µg/g 24h LD-50
Eumaeus atala [5]	Larval	Naled	Oral /feeding	0.206 µg/g 24h LD-50
Eumaeus atala [5]	Larval	Dichlorvos	Oral /feeding	0.206 µg/g 24h LD-50
Eumaeus atala [3]	Larval	Naled (diesel)	1 µl onto the dorsum of the thorax	0.0009 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Eumaeus atala [3]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.0009 LD-50 after 24 h in micrograms of active ingredient per gram of body weight

Eumaeus atala [4]	Adult	Permethrin	0.5 µl on each forewing	0.66 µg/g 24h LD-50
Eumaeus atala [4]	Adult	Naled	0.5 µl on each forewing	1.31 µg/g 24h LD-50
Eumaeus atala [4]	Adult	Dichlorvos	0.5 µl on each forewing	1.73 µg/g 24h LD-50
Eumaeus atala [4]	Adult	Permethrin	1 µl onto the dorsum of the thorax	1.60 µg/g 24h LD-50
Eumaeus atala [4]	Adult	Naled	1 µl onto the dorsum of the thorax	28.22 µg/g 24h LD-50
Eumaeus atala [4]	Adult	Dichlorvos	1 µl onto the dorsum of the thorax	6.56 µg/g 24h LD-50
Eumaeus atala [3]	Adult	Naled (diesel)	1 µl onto the dorsum of the thorax	0.0012 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Eumaeus atala [3]	Adult	Permethrin	1 µl onto the dorsum of the thorax	0.0036 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Heliconius charitonius [4]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.11 µg/g 24h LD-50
Heliconius charitonius [4]	Larval	Naled	1 µl onto the dorsum of the thorax	0.45 µg/g 24h LD-50
Heliconius charitonius [4]	Larval	Dichlorvos	1 µl onto the dorsum of the thorax	1.57 µg/g 24h LD-50
Heliconius charitonius [2]	Larval	Fenthion	1 µl onto the dorsum of the thorax	11.057 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Heliconius charitonius [2]	Larval	Fenthion	1 µl onto the dorsum of the thorax	10.433 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Heliconius charitonius [3]	Larval	Malathion	1 µl onto the dorsum of the thorax	8.127 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Heliconius charitonius [3]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.0015 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Heliconius charitonius [4]	Adult	Dichlorvos	0.5 µl on each forewing	1.34 µg/g 24h LD-50
Heliconius charitonius [4]	Adult	Permethrin	1 µl onto the dorsum of the thorax	0.18 µg/g 24h LD-50
Heliconius charitonius [4]	Adult	Naled	1 µl onto the dorsum of the thorax	0.9 µg/g 24h LD-50
Heliconius charitonius [4]	Adult	Dichlorvos	1 µl onto the dorsum of the thorax	1.56 µg/g 24h LD-50
Heliconius charitonius [3]	Adult	Malathion	1 µl onto the dorsum of the thorax	48.087 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Heliconius charitonius [3]	Adult	Permethrin	1 µl onto the dorsum of the thorax	0.0004 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Icaricia icarioides blackmorei [12]	Larval	Surfactant	No specific dose, just spray on leaves so probably also ingestion	No influence on survival, faster development time, no influence on biomass, no impact on morphology

Icaricia icarioides blackmorei [12]	Larval	Fluazifop-*p*-butyl	No specific dose, just spray on leaves so probably also ingestion	No influence on survival, faster development time, no influence on biomass, no impact on morphology
Icaricia icarioides blackmorei [12]	Larval	Sethoxydim	No specific dose, just spray on leaves so probably also ingestion	No influence on survival, faster development time, no influence on biomass, no impact on morphology
Junonia coenia [4]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.23 µg/g 24h LD-50
Junonia coenia [4]	Larval	Naled	1 µl onto the dorsum of the thorax	4.04 µg/g 24h LD-50
Junonia coenia [4]	Larval	Dichlorvos	1 µl onto the dorsum of the thorax	7.36 µg/g 24h LD-50
Junonia coenia [4]	Larval	Permethrin	Oral /feeding	0.755 µg/g 24h LD-50
Junonia coenia [4]	Larval	Naled	Oral /feeding	0.237 µg/g 24h LD-50
Junonia coenia [4]	Larval	Dichlorvos	Oral /feeding	0.327 µg/g 24h LD-50
Junonia coenia [4]	Adult	Permethrin	0.5 µl on each forewing	5.15µg/g 24h LD-50
Junonia coenia [4]	Adult	Naled	0.5 µl on each forewing	13.6 µg/g 24h LD-50
Junonia coenia [4]	Adult	Dichlorvos	0.5 µl on each forewing	5.99 µg/g 24h LD-50
Junonia coenia [4]	Adult	Permethrin	1 µl onto the dorsum of the thorax	1.07 µg/g 24h LD-50
Junonia coenia [4]	Adult	Naled	1 µl onto the dorsum of the thorax	6.84 µg/g 24h LD-50
Junonia coenia [4]	Adult	Dichlorvos	1 µl onto the dorsum of the thorax	11.3 µg/g 24h LD-50
Junonia coenia [7]	Adult	Naled	5 µl dose dorsal side of the thorax	4.9 µg/g 24h LD-50
Neophasia menapia [13]	Larval	SBP-138	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.013, LD-90: 0.058 oz/acre
Neophasia menapia [13]	Larval	Pyrethrins	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.037, LD-90: 0.11 oz/acre
Neophasia menapia [13]	Larval	Dewco-214	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.19, LD-90: 0.31 oz/acre
Neophasia menapia [13]	Larval	Methomyl	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.30, LD-90: 1.1 oz/acre
Neophasia menapia [13]	Larval	Chlorpyrifos	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.35, LD-90: 1.1 oz/acre
Neophasia menapia [13]	Larval	Tetrachlorvinphos	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.52, LD-90: 1.5 oz/acre
Neophasia menapia [13]	Larval	Sumithion	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.62, LD-90: 1.8 oz/acre
Neophasia menapia [13]	Larval	Phoxim	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.72, LD-90: 2.4 oz/acre

Neophasia menapia [13]	Larval	Zectran	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.32, LD-90: 2.8 oz/acre
Neophasia menapia [13]	Larval	Aminocarb	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 0.70, LD-90: 3.1 oz/acre
Neophasia menapia [13]	Larval	Malathion	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 1.8, LD-90: 4.1 oz/acre
Neophasia menapia [13]	Larval	Carbaryl	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 1.0, LD-90: 4.3 oz/acre
Neophasia menapia [13]	Larval	DDT	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50: 2.7, LD-90: 6.8 oz/acre
Neophasia menapia [13]	Larval	Trichlorfon	Ponderosa pine needles sprayed 0.0339 µl/cm2	Measured after 3 days LD-50:> 4.8 oz/acre
Papilio cresphontes [2]	Larval	Naled	1 µl onto the dorsum of the thorax	0.9 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Fenthion	1 µl onto the dorsum of the thorax	52.18 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Malathion	1 µl onto the dorsum of the thorax	28.65 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Resmethrin	1 µl onto the dorsum of the thorax	0.0021 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Naled	1 µl onto the dorsum of the thorax	0.966 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Fenthion	1 µl onto the dorsum of the thorax	193.010 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Malathion	1 µl onto the dorsum of the thorax	62.463 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Resmethrin	1 µl onto the dorsum of the thorax	0.0030 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Naled	1 µl onto the dorsum of the thorax	0.384 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Fenthion	1 µl onto the dorsum of the thorax	41.14 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Malathion	1 µl onto the dorsum of the thorax	128.455 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Larval	Resmethrin	1 µl onto the dorsum of the thorax	0.0023 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Papilio cresphontes [2]	Adult	Naled	1 µl onto the dorsum of the thorax	0.190 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)

Species	Stage	Compound	Method	Result
Papilio demoleus [14]	Larval	β-Asarone	Leaf dipped in 200, 150, 100, 50 ppm, feeding measured after 4h of starving	Significant anti-feeding activity at 200 pp, for 24h and 48h exposure
Papilio demoleus [15]	Larval	Diofenolan	7.5, 15, 30 and 60 µg µl^{-1} on posterior abdominal segment	Mortality, a range of developmental deformities, delayed larval-larval/pupal ecdysis and inhibition adult emergence
Papillo spp.[16]	Egg	BHC	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 0% at 0.025%
Papillo spp.[16]	Egg	Dicrotophos	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 86-90.8% at 0.025%
Papillo spp.[16]	Egg	Chlorfenvinphos	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 44-50% at 0.025%
Papillo spp.[16]	Egg	Carbaryl	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 56.3-78% at 0.025%
Papillo spp.[16]	Egg	Diazinon	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 33.3% at 0.025%
Papillo spp.[16]	Egg	Dichlorovos	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 66.6-90.8% at 0.025%
Papillo spp.[16]	Egg	Dimethoate	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 44-62.5% at 0.025%
Papillo spp.[16]	Egg	Formothian	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 27-50% at 0.025%
Papillo spp.[16]	Egg	Malathion	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 80-83% at 0.025%
Papillo spp.[16]	Egg	Methamidophos	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 100% at 0.025%
Papillo spp.[16]	Egg	Parathion	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 87.5% at 0.025%
Papillo spp.[16]	Egg	Phosphamidon	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 0% at 0.025%
Papillo spp.[16]	Egg	Quinalphos	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 93.8% at 0.025%
Papillo spp.[16]	Egg	Tricholorofon	Spray with 0.05 and 0.025% concentration	Moratality: 100% at 0.05% and 93-100% at 0.025%
Pieris brassicae [17]	Egg	Paraoxon	Leaf dipped in the different concentrations (0.005, 0.001, 0.0005, and 0.0001 %)	Egg survival and Cholinesterase activity measured, high Ach might be responsible to a failure to hatch
Pieris brassicae [18]	Larval	Deltamethrin	Topical application on dorsal surface	LD-50's and reduced larval weight
Pieris brassicae [18]	Larval	Deltamethrin	Exposed on leave disk	Percentages of mortality over days
Pieris brassicae [19]	Larval	Dimethoate	Single topical dose of insecticide on the abdomen of 0.25 µl	0.208 µg per insect 24h LD-50
Pieris brassicae [19]	Larval	Pirimicarb	Single topical dose of insecticide on the abdomen of 0.25 µl	0.158 µg per insect 24h LD-50
Pieris brassicae [19]	Larval	Phosalone	Single topical dose of insecticide on the abdomen of 0.25 µl	0.0109 µg per insect 24h LD-50
Pieris brassicae [19]	Larval	Endosulfan	Single topical dose of insecticide on the abdomen of 0.25 µl	6.46·10-3 µg per insect 24h LD-50

Pieris brassicae [19]	Larval	Fenitrothion	Single topical dose of insecticide on the abdomen of 0.25 µl	$1.18 \cdot 10^{-3}$ µg per insect 24h LD-50
Pieris brassicae [19]	Larval	Pirimiphos-methyl	Single topical dose of insecticide on the abdomen of 0.25 µl	$1.11 \cdot 10^{-3}$ µg per insect 24h LD-50
Pieris brassicae [19]	Larval	Fenvalerate	Single topical dose of insecticide on the abdomen of 0.25 µl	$5.39 \cdot 10^{-4}$ µg per insect 24h LD-50
Pieris brassicae [19]	Larval	Diflubenzuron	Single topical dose of insecticide on the abdomen of 0.25 µl	$2.5 \cdot 10^{-4}$ µg per insect 24h LD-50
Pieris brassicae [20]	Larval	Cypermethrin	Fed on a leaf dipped in pesticide	0.27 mg· L^{-1} LD-95 48h
Pieris brassicae [20]	Larval	Permethrin	Fed on a leaf dipped in pesticide	1.26 mg· L^{-1} LD-95 48h
Pieris brassicae [20]	Larval	Cypermethrin	Fed on a leaf dipped in pesticide	0.48 mg· L^{-1} -> 50% reduction in consumption, 2.55 mg· L^{-1} LD-50
Pieris brassicae [20]	Larval	Permethrin	Fed on a leaf dipped in pesticide	0.54 mg· L^{-1} -> 50% reduction in consumption, 3.6 mg· L^{-1} LD-50
Pieris brassicae [21]	Larval	Diflubenzuron	Pots downwind spray areas	Higher wind speeds, higher LD-50 distance
Pieris brassicae [22]	Larval	Deltamethrin	Fed on a leaf dipped in pesticide	0.4 µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	λ-cyhalothrin	Fed on a leaf dipped in pesticide	0.5 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Alphametrin	Fed on a leaf dipped in pesticide	1.0 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Bifenthrin	Fed on a leaf dipped in pesticide	1.1 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	β-cyfluthrin	Fed on a leaf dipped in pesticide	1.4 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Fenpropathrin	Fed on a leaf dipped in pesticide	1.2 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Cypermethrin	Fed on a leaf dipped in pesticide	9.0 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Fenvalerate	Fed on a leaf dipped in pesticide	16.0 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Deltamethrin	Sprayed with different concentrations	0.5 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	λ-cyhalothrin	Sprayed with different concentrations	0.8 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Alphametrin	Sprayed with different concentrations	1.1 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Bifenthrin	Sprayed with different concentrations	1.3 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	β-cyfluthrin	Sprayed with different concentrations	1.5 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Fenpropathrin	Sprayed with different concentrations	1.9 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Cypermethrin	Sprayed with different concentrations	11.6 in µg/ml LC-50 at 24h
Pieris brassicae [22]	Larval	Fenvalerate	Sprayed with different concentrations	19.0 in µg/ml LC-50 at 24h

Pieris brassicae [23]	Larval	DE / New silica	Fed on treated plants	Less leaf damage and weight gain in treated plants
Pieris brassicae [24]	Larval	Spinosad	Fed leaves treated with pesticide; 0.1 l/ha	Mortality 100% independent of instar
Pieris brassicae [24]	Larval	Cypermethrin	Fed leaves treated with pesticide; 0.3 l/ha	Mortality of caterpillars depended on larval instar and mixtures with other pesticides
Pieris brassicae [25]	Larval	Diazinon	2µl on the mesothoracic terga of the Larval	8.8 LD-50 (mg/kg) after 24h
Pieris brassicae [25]	Larval	Diazoxon	2µl on the mesothoracic terga of the Larval	11.0 LD-50 (mg/kg) after 24h
Pieris brassicae [26]	Larval	Cypermethrin	Topical application	0.00016 µg per insect 0.231 µg /g LD-50
Pieris brassicae [26]	Larval	Triazophos	Topical application	Larval age and their LD-50's Day 1 : 1.521 µg /g, Day 2: 03.103 µg/g, Day3: 3.283 µg /g
Pieris brassicae [26]	Larval	Dimethoate	Topical application	627 µg /g LD-50
Pieris brassicae [26]	Larval	Diflubenzuron	Topical application	0.87 µg /g LD-50
Pieris brassicae [26]	Larval	Triazophos/ Cypermethrin	Distance from sprayed cropped, so direct and by feeding	Mortality depends on spray distance and age of Larval
Pieris brassicae [27]	Larval	Dichlorvos	Pesticide sprayed on Petri dish, leaves and then Larval placed on it	0.0173004 % LC-50 at 24h
Pieris brassicae [27]	Larval	Endosulfan	Pesticide sprayed on Petri dish, leaves and then Larval placed on it	0.030497 % LC-50 at 24h
Pieris brassicae [27]	Larval	Quinolphos	Pesticide sprayed on Petri dish, leaves and then Larval placed on it	0.0496829 % LC-50 at 24h
Pieris brassicae [27]	Larval	Carbaryl	Pesticide sprayed on Petri dish, leaves and then Larval placed on it	0.0882649 % LC-50 at 24h
Pieris brassicae [28]	Larval	Pirimicarb	Topical application of 0.2 µl	0.0084 g/l LC-50 at 24h
Pieris brassicae [28]	Larval	Pirimicarb	Eating from a sprayed plant, 100, 10, 2 and 1% of the 0.42 g/l commercial application rate	LD-50 is around 30% of actual field dose
Pieris brassicae [28]	Larval	Diflubenzuron	Eating from a sprayed plant 100, 10, 2 and 1% of the 0.16 mg per plant application rate	LD-50-> 0.0034 mg/plant, around 1.9% of actual field dose
Pieris napi [29]	Larval	Dimethoate	Topical application / mimic spray drift	LD-50: 0.834 µg/per insect
Pieris napi [29]	Larval	Phosalone	Topical application / mimic spray drift	LD-50: 0.0686 µg/per insect
Pieris napi [29]	Larval	Fenitrothion	Topical application / mimic spray drift	LD-50: 0.0077 µg/per insect
Pieris napi [29]	Larval	Diflubenzuron	Topical application / mimic spray drift	LD-50: 0.0013 µg/per insect
Pieris rapae [12]	Larval	Surfactant	No specific dose, just spray on leaves so probably also ingestion	Lower survival, no influence on development time, no influence body mass, increase abdomen width

Species	Stage	Substance	Application	Effect
Pieris rapae [12]	Larval	Fluazifop-*p*-butyl	No specific dose, just spray on leaves so probably also ingestion	Lower survival, no influence on development time, no influence body mass, reduction wing size
Pieris rapae [12]	Larval	Sethoxydim	No specific dose, just spray on leaves so probably also ingestion	Lower survival, no influence on development time, no influence body mass, reduction wing size
Pieris rapea [18]	Larval	Deltamethrin	Topical application on dorsal surface	0.25 µl on dorsal surface drop. Different concentrations
Pieris rapea [30]	Larval	Pumpkin leaf acetone extract	Exposure to treated leaves after 4 hours of starving	It has good anti feeding effect at 700 mg/l
Polymmatus icarus [29]	Larval	Fenitrothion	Topical application / mimic spray drift	LD-50 0.024 µg/per insect
Polymmatus icarus [31]	Larval	Clothianidin	Eating from treated plant with a dose of 0, 5, 15, 50 or 500 ppb	Treatment reduces survival; larval growth is inhibited with 15 ppb or more. No effect on development time, pupal weight, adult weight or duration of the pupal stage
Proteus urbanus [3]	Larval	Naled (acetone)	1 µl onto the dorsum of the thorax	3rd instar :0.0699 LD-50 ; 4th instar: 0.0439 LD-50; 5th instar: 0.0296 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Proteus urbanus [3]	Larval	Malathion	1 µl onto the dorsum of the thorax	3rd instar: 0.2603 LD-50; 4th instar: 8.912 LD-50; 5th instar: 0.3045 after 24 h in micrograms of active ingredient per gram of body weight
Proteus urbanus [3]	Larval	Naled (diesel)	1 µl onto the dorsum of the thorax	0.0889 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Proteus urbanus [3]	Adult	Naled (acetone)	1 µl onto the dorsum of the thorax	0.1892 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Proteus urbanus [3]	Adult	Naled (diesel)	1 µl onto the dorsum of the thorax	0.3632 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Proteus urbanus [3]	Adult	Malathion	1 µl onto the dorsum of the thorax	13.458 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Pygrus oileus [3]	Larval	Naled (acetone)	1 µl onto the dorsum of the thorax	4th instar : 1.021 LD-50; 5th instar: 0.304 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Pygrus oileus [3]	Adult	Naled (acetone)	1 µl onto the dorsum of the thorax	0.0823 LD-50 after 24 h in micrograms of active ingredient per gram of body weight
Pyronia tithonus [29]	Larval	Fenitrothion	Topical application / mimic spray drift	LD-50 0.0273 µg/per insect
Pyronia tithonus [29]	Larval	Diflubenzuron	Topical application / mimic spray drift	LD-50 0.0051µg/per insect
Vanessa cardui [4]	Larval	Permethrin	1 µl onto the dorsum of the thorax	0.46 µg/g 24h LD-50

Vanessa cardui [4]	Larval	Naled	1 µl onto the dorsum of the thorax	10.82 µg/g 24h LD-50
Vanessa cardui [4]	Larval	Dichlorvos	1 µl onto the dorsum of the thorax	3.79 µg/g 24h LD-50
Vanessa cardui [2]	Larval	Naled	1 µl onto the dorsum of the thorax	0.417 LD-50-value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [2]	Larval	Fenthion	1 µl onto the dorsum of the thorax	70.673 LD-50-value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [2]	Larval	Malathion	1 µl onto the dorsum of the thorax	51.599 LD-50-value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [2]	Larval	Resmethrin	1 µl onto the dorsum of the thorax	0.1104 LD-50-value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [10]	Larval	Imidacloprid	Fed on plant grown on soil exposed to pesticides: 300 AI mg/pot or 600 AI mg/pot	Reduced survival in both conditions
Vanessa cardui [4]	Adult	Permethrin	0.5 µl on each forewing	8.69 µg/g 24h LD-50
Vanessa cardui [4]	Adult	Naled	0.5 µl on each forewing	2.29 µg/g 24h LD-50
Vanessa cardui [4]	Adult	Dichlorvos	0.5 µl on each forewing	6.68 µg/g 24h LD-50
Vanessa cardui [4]	Adult	Permethrin	1 µl onto the dorsum of the thorax	1.10 µg/g 24h LD-50
Vanessa cardui [4]	Adult	Naled	1 µl onto the dorsum of the thorax	30.08 µg/g 24h LD-50
Vanessa cardui [4]	Adult	Dichlorvos	1 µl onto the dorsum of the thorax	4.66 µg/g 24h LD-50
Vanessa cardui [2]	Adult	Naled	1 µl onto the dorsum of the thorax	0.541 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [2]	Adult	Fenthion	1 µl onto the dorsum of the thorax	5.848 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [2]	Adult	Malathion	1 µl onto the dorsum of the thorax	10.719 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [2]	Adult	Resmethrin	1 µl onto the dorsum of the thorax	0.0067 LD-50 value in µg active ingredient/ g bodyweight (24h after exposure)
Vanessa cardui [10]	Adult	Imidacloprid	300 or 600 AI mg/pot (natural) or 0.15g or 0.3g in honey solution	No reduced survival
Vanessa cardui [7]	Adult	Naled	5 µl dose dorsal side of the thorax	5.1 µg/g 24h LD-50

Phylogenetic analyses

1. Multidrug resistance (mdr) genes

The *mdr* genes, a group of related duplicated genes belonging to the ABC transporter superfamily (Dermauw and Van Leeuwen, 2014; Tapadia and Lakhotia, 2005), play a significant role in the defence against a range of different harmful compounds. Differential gene expression levels as well as sequence variation in these *mdr* genes have been shown to be the cause of population differences in the response to toxic compounds, and the development of resistance (Dermauw and Van Leeuwen, 2014). An example of this includes the resistance to Deathcap mushroom toxicity in a *D. melanogaster* population by means of *mdr65* (Begun and Whitley, 2000). The phylogenetic tree in supplementary figure 1 shows that *mdr65* is a Dipteran paralog of *mdr49* that diverged significantly from *mdr49*. Lepidoptera, on the other hand appear not to have *mdr65*, but another paralogous cluster to *mdr49*, which appears to represent at least 2 unique paralogs. At present, we do not know whether *mdr* genes, and in particular these unique paralogs, play a role of significance in differences in sensitivity to harmful compounds between Lepidopteran populations, let alone butterflies (Simmons et al., 2013). In terms of expression patterns, these genes appear to be expressed throughout development in *D. melanogaster*, from early embryos to adults and in a variety of tissues (Fisher et al., 2012). However, there are some differences between life-stages and individual *mdr* genes. For example, *mdr65* has very low expression levels in third instar *D. melanogaster* larvae and older females and is not maternally provided in eggs. Transcripts of *mdr49*, on the other hand, are present throughout *Drosophila* development, including maternal transcripts.

2. Ryanodine receptors

Ryanodine receptors are a class of intracellular calcium channels that are targets for a recently developed class of insecticides known as diamides (Lahm et al., 2005; Sattelle et al., 2005; Sparks and Nauen, 2015). Unlike *mdr* genes, these have been studied intensively in Lepidoptera, be it only in moths (Gong et al., 2014; Guo et al., 2014; Sun et al., 2015) . In moths, they have been shown to be associated with (large) population differences in sensitivity to pesticides, as well as pesticide resistance; for example, to various diamides (Bird, 2016; Troczka et al., 2012; Troczka et al., 2015; Wu et al., 2014; Yao et al., 2016). Such population differences in sensitivity, which have a strong ecological significance (Steinbach et al., 2015), are quite often the result of simple point mutations making them a less effective target for the relevant pesticides (Guo et al., 2014). Rather interestingly, RyR genes are not as variable as *mdr* genes, and no unique duplications in the Lepidoptera can be observed (supplementary figure 2). Like *mdr65*, *RyR* is also expressed throughout development in *D. melanogaster*, in particular in muscle tissue, and being absent as maternal transcripts and in the final instar (Fisher et al., 2012; Hasan and Rosbash, 1992). Such expression data profiles, for which we only have sufficient data on *D. melanogaster*, do indicate that different life-history stages are likely to display different sensitivities to pesticide use, and which is something that studies on butterflies will need to take firmly into account.
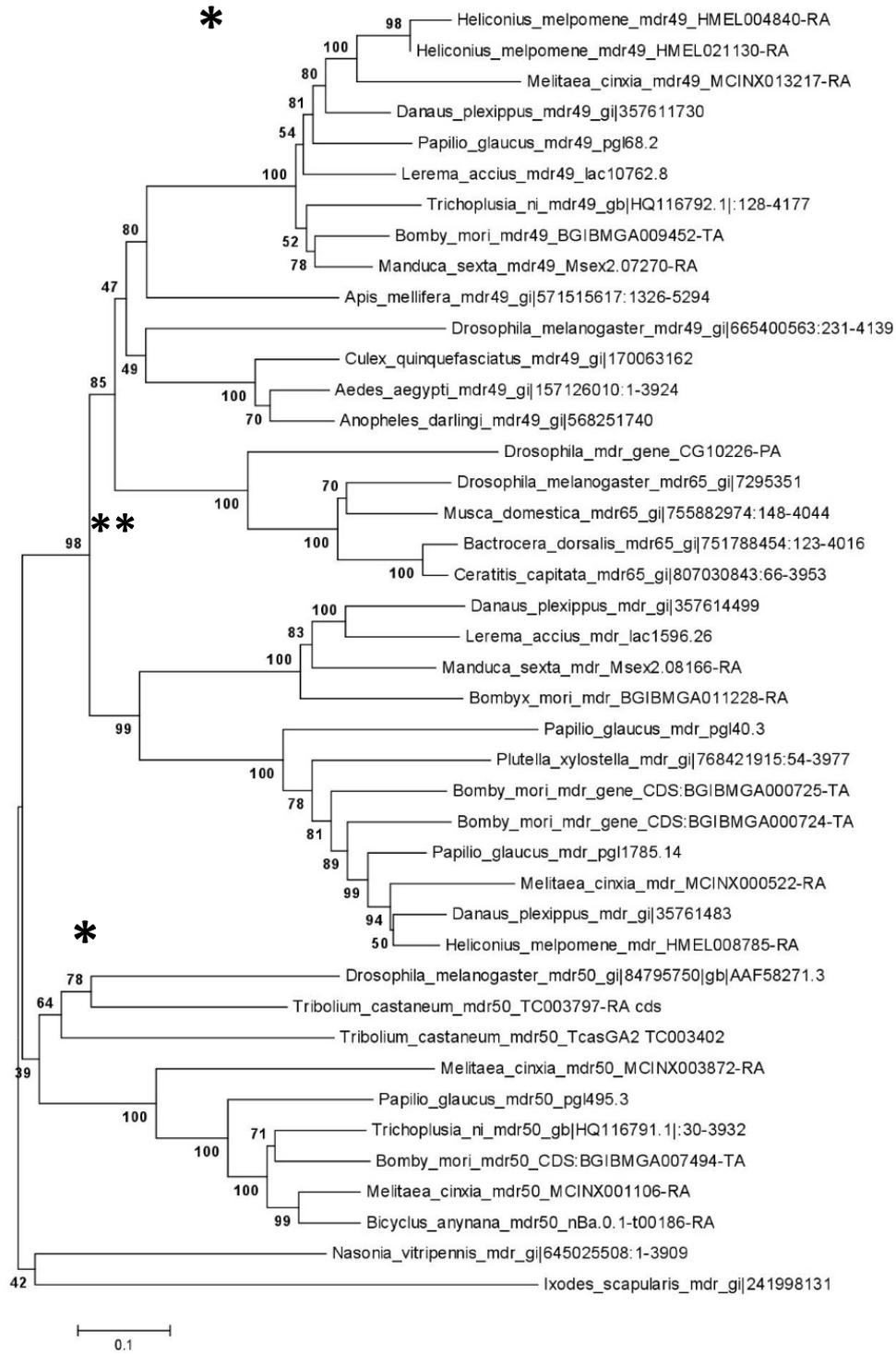
Figure 1. Phylogenetic reconstruction for the evolution of the insect *mdr* gene family, with an emphasis on Lepidoptera (clades indicated with asterisk; ** indicates a clade with unique paralogs for Lepidoptera), inferred using a Neighbor-Joining method conducted in MEGA6 (Saitou and Nei, 1987; Tamura et al., 2013). The analysis took place on 42 amino acid sequences, eliminating sites with less than 95% coverage including those

with missing data, ambiguous bases and alignment gaps. The final dataset totalled 642 positions and the resultant *mdr* tree has a branch length sum of 7.93074509. The values situated on nodes and branches detail the percentage of replicate trees in which the associated taxa clustered together in the 500 replicate bootstrap test (Felsenstein, 1985). The evolutionary distances, corresponding to the branch lengths, were calculated using the Poisson correction method using the same units originally used to infer the phylogenetic tree (Zuckerkandl and Pauling, 1965). Branch lengths are therefore drawn to scale.

Figure 2. Phylogenetic reconstruction of the insect RyR gene family, with an emphasis on Lepidoptera (indicated with asterisk), inferred using a Neighbor-Joining method conducted in MEGA6 (Saitou and Nei, 1987; Tamura et al., 2013). The analysis took place on 33 amino acid sequences, eliminating sites with less than 95% coverage including those with missing data, ambiguous bases and alignment gaps. The final dataset totalled 4596 positions and the resultant *mdr* tree has a branch length sum of 2.28881154. The values situated on nodes and branches detail the percentage of replicate trees in which the associated taxa clustered together in the 500

replicate bootstrap test (Felsenstein, 1985). The evolutionary distances, corresponding to the branch lengths, were calculated using the Poisson correction method using the same units originally used to infer the phylogenetic tree (Zuckerkandl and Pauling, 1965). Branch lengths are therefore drawn to scale.

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