Thermal plasticity and sensitivity to insecticides in populations of an invasive beetle: Cyfluthrin increases vulnerability to extreme temperature

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J.E.D. and D.R. designed the experiments, carried out by J.E.D. and S. L. M.. D.R. and M.H. supervised the project. J.E.D. and D.R. derived the models and J.E.D. and S.L.M. analysed the data. H.C. and D.R. assisted with data analysis. J.E.D. and D.R. wrote the manuscript in consultation with all authors. All authors discussed the results and contributed to the final manuscript.

Abstract

Climate change increases average temperatures and the occurrence of extreme weather events, in turn accentuating the risk of organism exposure to temperature stress. When thermal conditions become stressful, the sensitivity of insects toward insecticides can be exacerbated. Likewise, exposure of insects to insecticides can subsequently influence their ability to handle stressful temperatures. Here, we investigated the effects of constant temperature and daily heat spikes, in presence/absence of insecticide treatment (cyfluthrin), on the condition (impairment of mobility) and thermal tolerance to cold (- 6 °C) and heat (42.5 °C) of the terrestrial beetle *Alphitobius diaperinus*. The responses of insects from four populations (three farm-collected populations, one laboratory population) to different durations of extreme temperature exposure were compared. The results showed that the laboratory population was generally more sensitive to extreme cold and heat temperatures, with less than 50% of adults recovering after an exposure at -6 or +42.5 °C for 3h. Significant differences in the level of thermal tolerance were also found among insects from poultry farms. Cyfluthrin exposure incurred detrimental effects to insects' condition in all but one population. For two out of the four populations, mobility impairment was increased when adults were exposed to daily heat spikes (6 hours per day at 38 °C) and cyfluthrin simultaneously, compared to cyfluthrin exposure at constant temperatures; yet, no significant interaction between the two stressors was found. Finally, using one farm collected population, effects of pre-exposure to cyfluthrin on extreme temperature tolerance provided another example of the toxicant-induced climate sensitivity in insects.

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16   1. **Introduction**

17   Over the course of their life, all living organisms, including insects, are confronted with a range of

18   environmental conditions which vary spatially and temporally (Hirose and Nakamura 2005; Mann,

19   Bradley, and Hughes 1998; Soranno et al. 2019). In some instances, environmental constraints may

20   be exacerbated by the currently changing climatic conditions. This increases the occurrence of

21   extreme weather events and the probability of organisms' exposure to higher temperatures

22   (Hansen, Sato, and Ruedy 2012; IPCC 2014; Stillman 2019), heightening the risk of organism

23   exposure to heat stress. In turn, several life history traits of individual insects can be affected

24   (Bozinovic et al. 2011; Kingsolver, Diamond, and Buckley 2013; Renault 2011), with cascading effects

25   on population demography and organism evolution (Chown et al. 2010; Colinet et al. 2015; Katz and

26   Brown 1992; Moe et al. 2013). When temperature varies, heat spikes are more likely to occur, and

27   they are expected to have greater evolutionary significance - and pose greater threat to species

28   performance - than increase in average temperature (Katz and Brown 1992; Vasseur et al. 2014).

29   Yet, whatever the nature of the physiological and biochemical mechanisms triggered by thermal

30   stress, their activation may weaken the ability of organisms to deal with other environmental

31   constraints, or conversely magnify these abilities when the responses elicit cross tolerance

32   phenomena (Chen and Stillman 2012; Noyes and Lema 2015; Sinclair et al. 2013). In this context,

33   investigating insects' ability to cope with stress under and after exposure to varying temperature

34   regimes provides useful information about their sensitivity to environmental stress, and for future

35   insights into their geographic distribution (Bozinovic and Pörtner 2015; Buckley et al. 2010; Ma,

36   Hoffmann, and Ma 2015).

37   Besides climatic conditions, adverse environmental effects on insects are also caused by the

38   unintentional spread of man-made contaminants, in particular by the use of various pesticides in

39   agriculture and horticulture (Aviles et al. 2019; Calow 1991; Köhler and Triebskorn 2013). The use of

40   pesticides has become widespread in many regions of the world where agricultural intensity has

41   increased (Tilman et al. 2002), representing a threat to biodiversity in general. While valuable

42   insights of the effects of insecticides on behaviour (Dewer et al. 2016), olfaction (Lalouette et al.

43   2016), reproduction and development (Abbes et al. 2015) have been gathered, these investigations

44   were commonly conducted by exposing the organisms to constant and optimal temperatures, as

45   prescribed in standardized tests (Camp and Buchwalter 2016; Khan and Akram 2014). Yet, the

46   inclusion of daily thermal fluctuations is needed to better simulate the conditions experienced by

47   free-ranging organisms in natural and anthropogenic environments, and thus increase the quality

48   and realism of the outcomes of these investigations (Colinet et al. 2015; Verheyen and Stoks 2019).

49   Moreover, thermal tolerance can vary significantly among insect populations originating from

50   different environments (Engell Dahl et al. 2019; Käfer et al. 2020), as is also the case for pesticide

51   exposure (see Hawkins et al. (2019) for a review of the different mechanisms that may confer

52   different level of insecticide resistance among insect populations). When assessing the effects of

53   environmental stressors on insects' ecology, it has thus become crucial to consider multiple

54   populations (of different exposure or adaptation history) in the experimental designs to get a larger

55   coverage of the range of responses that could be expected at the species level.

56   The possible combined effects of climate change and environmental pollution on organisms, and

57   wildlife in general, have been reviewed by Noyes and Lema (2015). These authors reported that

58   chemical exposure may affect organisms' sensitivity to climate change by disrupting physiological

59   responses to diurnal and seasonal thermal fluctuations. In particular, the alteration of endocrine

60   system functioning and energy balance in toxicant-exposed organisms may subsequently alter their

61   thermal performance curves, potentially challenging their ability to survive thermal stress. In several

62   instances, it has been suggested that the simultaneous exposure to pollutants, including endocrine

63   disrupting chemicals, and climate change could result in synergistic actions (Cairns, Heath, and

64   Parker 1975; Hooper et al. 2013; Laskowski et al. 2010; Moe et al. 2013). When sequential

65   exposures occur, climatic stress can influence sensitivity towards pollutants, and the reverse is also

66   true, e.g. previous exposure to a pollutant can influence the tolerance to climatic stress (Hooper et

67   al. 2013).

68   Nowadays, studies assessing the impacts of multiple stressors, including temperature, are

69   increasingly needed (Kaunisto, Ferguson, and Sinclair 2016; Orr et al. 2020), as such investigations

70   greatly improve our estimates of the effects of interactions among environmental stressors on

71   insects. Holmstrup et al. (2010) reviewed the available studies that assessed the combined effects of

72   pesticide and temperature exposures, and reported only few works that considered daily thermal

73   fluctuations, or daily heat spikes, in their experimental designs. Since this review has been

74   published, the number of studies that combined temperature and toxicant exposures has started to

75   accumulate, reporting how thermal conditions could modify the effects of pesticides on insects (Op

76 de Beeck, Verheyen, and Stoks 2018; Camp and Buchwalter 2016; Delnat et al. 2019; Harwood, You,

77 and Lydy 2009; Verheyen, Delnat, and Stoks 2019; Verheyen and Stoks 2019; Willming, Qin, and

78 Maul 2013). For example, Verheyen and Stoks (2019) found that exposure of damselfly larvae to the

79 organophosphate chlorpyrifos did not affect the survival of individuals maintained at constant 20 °C.

80 Conversely, the mortality of the larvae was increased under daily thermal fluctuations of 5 (17.5-

81 22.5 °C) or 10 °C (15-25 °C). Delnat et al. (2019) reported that daily thermal fluctuations of 7 (16.5-

82 23.5 °C) or 14 °C (13-27 °C) slightly increased the mortality of the larvae of the mosquito *Culex*

83 *pipiens* exposed to chlorpyrifos, while having no effect on adults. Working with heat waves

84 (constant heat exposure of damselfly larvae over three consecutive days), Arambourou and Stoks

85 (2015) reported that the subsequent exposure to chlorpyrifos did not change mortality rates of the

86 larvae. However, the higher inhibition of acetylcholine esterase suggested that heat waves may

87 increase the sensitivity of damselflies to this chemical. More recent studies have examined the

88 effects of successive heat spikes and pesticide exposure (Meng, Delnat, and Stoks 2020), and the

89 delayed effects of pesticide exposures on life histories of insects (Dinh, Janssens, and Stoks 2016).

90 Even though Delnat, Janssens, and Stoks (2019) found the effect of warming on pesticide toxicity to

91 be dependent on other factors of their experimental setup, results from the available literature

92 generally underline the importance of incorporating more realism into the experimental designs

93 when investigating the interaction between pesticide and temperature (Böttger, Schaller, and Mohr

94 2012). Moreover, all but one of these studies used insect larvae, and all investigations were based

95 on aquatic species, meaning that terrestrial insect species and adult life stages are severely

96 underrepresented from this research. Likewise, some studies have investigated the thermal

97 tolerance of insects after they were exposed to pesticides (Op de Beeck, Verheyen, and Stoks 2017,

98 2018; Delnat et al. 2019; Meng, Delnat, and Stoks 2020). Yet again, all of these investigations have

99 focused on aquatic larvae, and used the organophosphate chlorpyrifos. Other chemicals, including

100 pyrethroids, which are also highly toxic to insects (Tang et al. 2017), should be considered. Finally,

101 no study examined the effects of sequential exposure to daily thermal fluctuations and pesticide on

102 the subsequent thermal tolerance in terrestrial insects.

103 In order to contribute to the abovementioned research gaps concerning the interaction of climate

104 and pollutant stress, we aimed at investigating the effects of daily heat spikes and insecticide

105 treatment on the thermal tolerance of adults of the lesser mealworm *Alphitobius diaperinus*

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106 (Panzer) (Coleoptera: Tenebrionidae), as well as comparing the performance of populations of *A.*
107 *diaperinus* of different exposure histories to temperature and insecticide. This insect pest,
108 pullulating in poultry farms from many regions worldwide (Geden and Hogsette 1994; Johnson,
109 Gbon, and Boga 2018; Rueda and Axtell 1997), represents a fitting model for research combining
110 ecophysiological and ecotoxicological investigations. Insects can be collected in large numbers from
111 geographically distant and unconnected habitats (poultry farms), making it possible to work with
112 different and locally adapted strains. In these anthropogenic environments, the lesser mealworms
113 are exposed to fluctuating temperatures, as broilers optimize poultry production by running a
114 standardized cycle (which has to be closely followed to maintain profitability (Donald 2010)) of
115 weekly decreasing temperatures, i.e. starting from ≈ 31 and reaching ≈ 17 °C over approximately
116 seven weeks (Salin, Delettre, and Vernon 2003). This provides an excellent habitat for lesser
117 mealworms, that have been found to grow well, i.e. with a very high energy conversion efficiency at
118 23 to 31 °C (Bjørge et al. 2018). Temperature extremes up to 48 °C have also been measured in the
119 litter where *A. diaperinus* thrive (Salin and Vernon 1997), meaning that the insect can be exposed to
120 heat spikes. The poultry houses are disinfected from time to time with chemicals, including the
121 structural type II pyrethroid, cyfluthrin (the use of this insecticide will be banned in France from July
122 21st, 2021 (ANSES 2020)). This insecticide is an inhibitor of insect nerve cell sodium channels, leaving
123 them open and causing paralysis, tremors, and eventually death (Lushchak et al. 2018). It is sprayed
124 at the end of each broiler flock for controlling outbreaks of *A. diaperinus*, and during the disinfection
125 and cleaning of the poultry farm, the heating system is stopped, doors of the building are left
126 opened, and temperature can vary from -5 °C to 31 °C (Salin, Delettre, and Vernon 2003).

127 As a first part of the study, we compared the basal thermal tolerance of *A. diaperinus* from four
128 populations, by measuring their ability to recover from exposure to extreme cold or heat
129 temperatures for different durations. We hypothesized that the beetles' recovery from thermal
130 stress would be faster in populations that experienced temperature fluctuations in their original
131 environment, as previously reported on several other insect models (Arias, Poupin, and Lardies
132 2011; Bozinovic et al. 2011; Cavieres et al. 2019). As a second part of the study, we were interested
133 in comparing the effects of exposure to an environmentally relevant dose of the insecticide
134 cyfluthrin on the condition of insects maintained at constant temperatures or subjected to daily
135 heat spikes. We hypothesized that populations which had experienced frequent pesticide exposure

would perform better than laboratory-reared adults (Hamm et al. 2006; Lambkin and Rice 2009). Finally, the population with the highest pesticide tolerance was exposed to different temperature regimes, without pesticide exposure or with different concentrations of cyfluthrin (up to those recommended by commercial formulations). Subsequently, the recovery times of individuals from the different treatments after an exposure to temperature extremes were assessed. Insects pre-exposed to daily heat spikes were expected to recover faster from heat shock, but slower from cold shock treatments, compared to insects kept at constant temperature. Furthermore, insects exposed to insecticides were hypothesized to be characterized by a lower ability to cope with temperature stress, either due to an increased toxicity of the chemical with daily heat spikes or decreased ability to cope with heat shock after chemical stress (Op de Beeck, Verheyen, and Stoks 2017; Delnat et al. 2019; Meng, Delnat, and Stoks 2020).

2. Materials and methods

2.1. Rearing of the insects

Adults of *A. diaperinus* were collected from three poultry farms in Brittany, France, thereafter referred to as FARM1 (sampled at Miniac-sous-Bécherel, 48°17'10'' N, 1°55'51'' W), FARM2 (sampled at Plaine Haute, 48°26'44'' N, 2°51'16'' W), and FARM3 (sampled at Pommerit-le-Vicomte, 48°37'10'' N, 3°05'15'' W). In addition, larvae of *A. diaperinus* were obtained from Proti-Farm *via* a pet shop (Envies Animales, Saint-Jacques-de-la-Lande, Brittany, France). Insects from this latter population, hereafter referred to as LAB, were reared over many generations under controlled conditions (25 °C, relative humidity 50%, no light) in a supposedly pollutant free environment. Because LAB insects are produced and used as a dietary food supply for pets (e.g. lizards), they are in very good physiological condition (Bjørge et al. 2018).

All adults were kept at 25 °C, in darkness, in plastic containers with sawdust for minimum 4 weeks prior to experimental start. They were supplied with water and food *ad libitum*, in the form of water in microtubes with cotton and dry dog food pellets. This procedure allowed standardisation of the temperature, humidity, and trophic conditions experienced by the adults before the assays. The LAB larvae were additionally provided with a block of polystyrene to burrow in for pupation, and oat bran for the first four weeks to further encourage their development into adults (Rice and Lambkin 2009). The sex and age of the adults was not considered in our experiments.

165   **2.2. Experimental design**

166   **2.2.1. Characterization of the thermal tolerance of the insects from different populations**

167   In order to compare the thermal tolerance of the insects from the four populations, adults were

168   exposed to two extreme temperatures, -6 or +42.5 °C, in a cryothermostat (VWR Collection, AP15R-

169   30). This procedure is derived from protocols commonly used for assessing the thermal tolerance in

170   insects (Hazell et al. 2008; Sinclair, Coello Alvarado, and Ferguson 2015). At -6 and + 42.5 °C, the

171   lesser mealworms are expected to fall into coma (see **Supplementary File 1** for the results of the

172   preliminary tests that allowed us designing the methodology). Each insect was exposed to one of

173   these two extreme temperatures for one of six durations: 15 min, 30 min, 1h, 2h, 3h or 4h, in

174   darkness. This range of stress durations allowed us to describe and compare thermal tolerance

175   among populations. At least four replicates of 10 adults (test tube with rubber cap) were used for

176   each exposure duration and temperature combination. At the end of the thermal treatment (-6 or

177   +42.5 °C), each group of 10 insects was directly transferred into Petri dishes and observed for up to

178   40 seconds every 5 minutes over 2h at room temperature (19 ± 1 °C) **(Supplementary File 2)**. During

179   each observation period, the recovery of the insects was scored by registering the individuals as

180   either "Active" or "Impaired/immobile"; insects were characterized as "Active" when they exhibited

181   coordinated movements of all limbs when stimulated, all other states were registered as

182   "Impaired/immobile". The temperatures, both in the cryothermostat and in the room where

183   recovery observations took place, were monitored using iButtons® (iButtonLink, LLC., Whitewater,

184   USA).

185   **2.2.2. Effect of daily heat spikes and cyfluthrin exposure**

186   Here, we combined two abiotic stresses by jointly exposing adult *A. diaperinus* to both moderately

187   stressful heat spikes and insecticide for eight days, before assessing any visible impairment or death

188   of exposed individuals. One temperature cabinet was set to constant 26 °C (control temperature),

189   and another cabinet varied the temperature by holding a 6h period at 38 °C, followed by 18h at 26

190   °C **(Supplementary File 2)**; the two cabinets were MIR 154 Panasonic programmable incubators. The

191   experiment was run in dark conditions.

192   Insects were placed in glass Petri dishes (10 cm diameter) with a fitting filter paper covering the

193   bottom. For insecticide application, cyfluthrin (pure molecule standard, CAS-number 68359-37-5,

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194   Sigma-Aldrich) was dissolved in acetone, and a volume of 2 mL of appropriately diluted solution was

195   transferred to the filter paper. Pre-testing the insects' susceptibility to cyfluthrin led to preparation

196   of two distinct insecticide concentrations, so that the applied dose was 20 mg cyfluthrin/m^2 for

197   populations FARM1, 2 and 3, and 0.5 mg cyfluthrin/m^2 for LAB. We had to reduce the cyfluthrin

198   dose significantly for the LAB population, as the 20 mg cyfluthrin / m² concentration killed all adults

199   within a day at our experimental temperatures. These cyfluthrin concentrations induced negligible

200   knockdown (<5%) over prolonged exposures in the works of Desvignes-Labarthe (2018) and Renault

201   and Colinet (Submitted). These concentrations also range in the values that may be encountered by

202   the insects in poultry houses (Salin, Delettre, and Vernon 2003). In the other Petri dishes, 2 mL of

203   pure acetone was added to control for any effect of the solvent. All Petri dishes were left to dry

204   under a fume hood for at least 10 min before the tests, which effectively evaporated all acetone.

205   The exiting literature reveals that cyfluthrin remains efficient over seven days (Guillebeau, All, and

206   Javid 1989), with knockdown effects remaining unchanged when the beetle *Tribolium castaneaum*

207   were assessed eight weeks after the chemical was applied (Arthur 1999) Finally, starting from an

208   initial concentration of 17.3 mg / m², (Nakagawa et al. 2017) reported that cyfluthrin concentration

209   decreased by ca. 17% after 56 days. Thus, even if changes in cyfluthrin concentration were not

210   monitored here, it is assumed that it remained close to the nominal concentration.

211   For all treatments, the insects were supplied with a water source (0.5 mL microtube containing

212   water and cotton) and a food source (approx. 0.8 g dog food pellet). For each population, a set of

213   Petri dishes treated with the insecticide and a set of dishes without pesticide treatment were placed

214   in the temperature cabinets (8 to 30 dishes of each, as specified in Table 1) along with an iButton®

215   (iButtonLink, LLC., Whitewater, USA) measuring both temperature and humidity. Each Petri dish in

216   experiments with poultry house collected populations contained 30 individuals, and for the LAB

217   population, each Petri dish contained 20 individuals, due to limited number of individuals **(Table 1)**.

218   Previous experiments that examined the sensitivity of *A. diaperinus* to insecticides (including

219   cyfluthrin) revealed that densities from 10 to 30 adults per Petri dish had no effects on the results

220   (Desvignes-Labarthe 2018; Renault and Colinet Submitted).

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224 *Table 1 Overview of number of dishes and number of individuals per dish used for assessing the*
225 *effects of temperature regime (constant temperature or daily heat spikes), with and without the*
226 *insecticide cyfluthrin, on the locomotory capacities of adult* Alphitobius diaperinus*.*

227

Treatment	**Without insecticide**		**Insecticide-exposed**	
Population	Dishes (N)	Animals (N)	Dishes (N)	Animals (N)
FARM1	20	30	20	30
FARM2	21	30	30	30
FARM3	10	30	10	30
LAB	8	20	12	20

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230 After eight days, and 17 hours after the last peak of heat, the state of each individual was noted: the

231 insects were considered as either "Active" (no apparent effect of the treatment on the locomotory

232 activities of the insects), "Impaired" (locomotor deficits: difficulties in moving limbs, or laying on

233 their backs while moving) or "Immobile" (no observable movement).

234 **2.2.3. Effect of daily heat spikes and cyfluthrin exposure on recovery from exposure to extreme**

235 **temperatures**

236 In the third part of the study, we measured the capabilities of adult *A. diaperinus* to recover from

237 cold or heat shocks after they had been exposed to insecticide and/or daily heat spikes for eight

238 days. As a non-negligible part of the adults from LAB, FARM2 and FARM3 were impaired or

239 immobile after they were exposed to cyfluthrin concentration of 20 mg / m² (see the results

240 presented in Figure 3), we had to focus on adults from FARM1 for this experiment. Lesser

241 mealworms from this population survive exposure to cyfluthrin concentrations encountered in the

242 field (as recommended applications contain 20 mg cyfluthrin/m², C. Salin et al., 2003), which thus

243 allowed working with this concentration without incurring selection of individuals due to mortality

244 or locomotor deficits during the pre-exposure period.

245 For this experiment, three temperature conditions were used: (i) constant 26 °C for eight days

246 (control), 18h at 26 °C daily interrupted by exposures of 6h at (ii) 32 °C (low heat spike), or (iii) 38 °C

247 (high heat spike). The exposure ran over eight days in darkness. Glass Petri dishes were prepared

248 with four doses of cyfluthrin (0, 5, 10 or 20 mg cyfluthrin/m²) as described above, including addition

249 of food and water. Then, 30 adults from FARM1 were added to each dish, and dishes of every

250 pesticide dose were placed into each of the three temperature cabinets (MIR 154 Panasonic

251   Incubators), resulting in a total of four different pesticide exposures at three different temperature

252   regimes.

253   After eight days, and 17 hours after the last peak of heat, the Petri dishes were removed from the

254   temperature cabinets, and the beetles were directly exposed to either -6 or +42.5 °C for 2h, in

255   darkness **(Supplementary File 2)**. Extreme temperature exposure was performed as described

256   above (section 2.2.1.), including testing the recovery of at least 40 individuals to each combination

257   of treatment (temperature regime, insecticide dose, and temperature regime x insecticide dose),

258   but the temperature of the recovery room was higher (approx. 24 °C instead of 20 °C).

259   **2.3. Data treatment**

260   The recovery time of every single individual was used to compute the temporal recovery curves

261   using Kaplan Meier estimates computed in R version 3.5.2, using the Survminer package

262   (Kassambara and Kosinski 2018; R Core Team 2013). The probability of recovery at any given time

263   point therefore represents the proportion of individuals that have recovered by that time. Pairwise

264   comparisons using Log-Rank test with adjusted P-values (Benjamini and Hochberg 1995) were

265   performed *post-hoc*, with a significance value of 0.05. Generalized linear models (GLMs) with logit

266   link function for proportions outcome (i.e. number of active versus impaired or immobile individuals

267   per dish) were run on data reporting the state of animals after eight days of exposure to the

268   different temperature regimes and pesticide doses. The effect of each variable (temperature, and

269   insecticide dose), and their interactions, were analysed using the analysis of deviance (Anova

270   function in the car package (Fox and Weisberg 2019)). We assume that this insecticide and heat

271   peaks have different modes of actions, meaning that their individual- and interaction effects on *A.*

272   *diaperinus* adults are assumed to be multiplicative. Differences among the treatments were finally

273   analysed by Sidak's multiple comparisons using the emmeans and multcompView packages (Graves,

274   Piepho, and Selzer 2019; Lenth 2020), with a significance value of 0.05.

275   **3. Results**

276   **3.1. Characterization of the thermal tolerance of the insects from different populations**

277   The recovery probability over time of adult *A. diaperinus* was dependent on the exposure duration

278   to hot or cold temperature extremes **(Figures 1 and 2; Supplementary File 3)**. Adults from LAB

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recovered more slowly than adults from the poultry house-collected populations for all durations of exposure at 42.5 °C (P ≤ 0.05), and all but 30 minutes exposure at -6 °C (P ≤ 0.05) **(Supplementary File 3)**. After a 30 min exposure at -6 °C, the recovery from LAB was comparable to that measured from the three other populations (P ≥ 0.05); after 1 hour exposure at 42.5 °C, the recovery curve of LAB was also similar to FARM3 (P = 0.33). When the duration of exposure to heat or cold temperatures was increased, the proportion of insects that fully recovered was decreased and the duration of recovery was prolonged **(Figures 1 and 2)**.

The recovery probability of the lesser mealworms from FARM1 and FARM2 after they were exposed to heat (42.5 °C) was similar for all experimental conditions. When exposed to temperature extremes for 0.25, 0.5, 1, 2, or 3h, insects from FARM3 were characterised by a lower recovery probability as compared with the two other poultry house-collected populations (Figure 1).
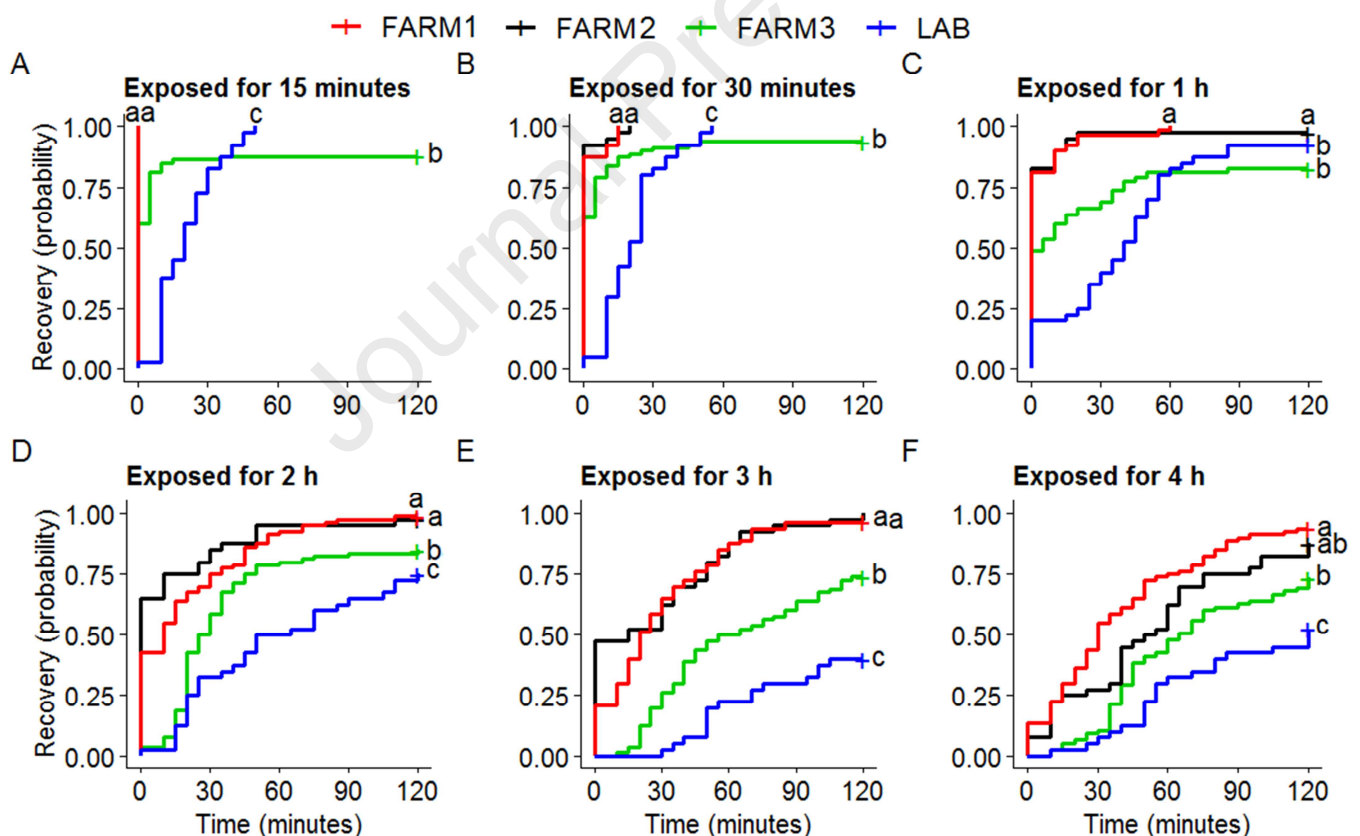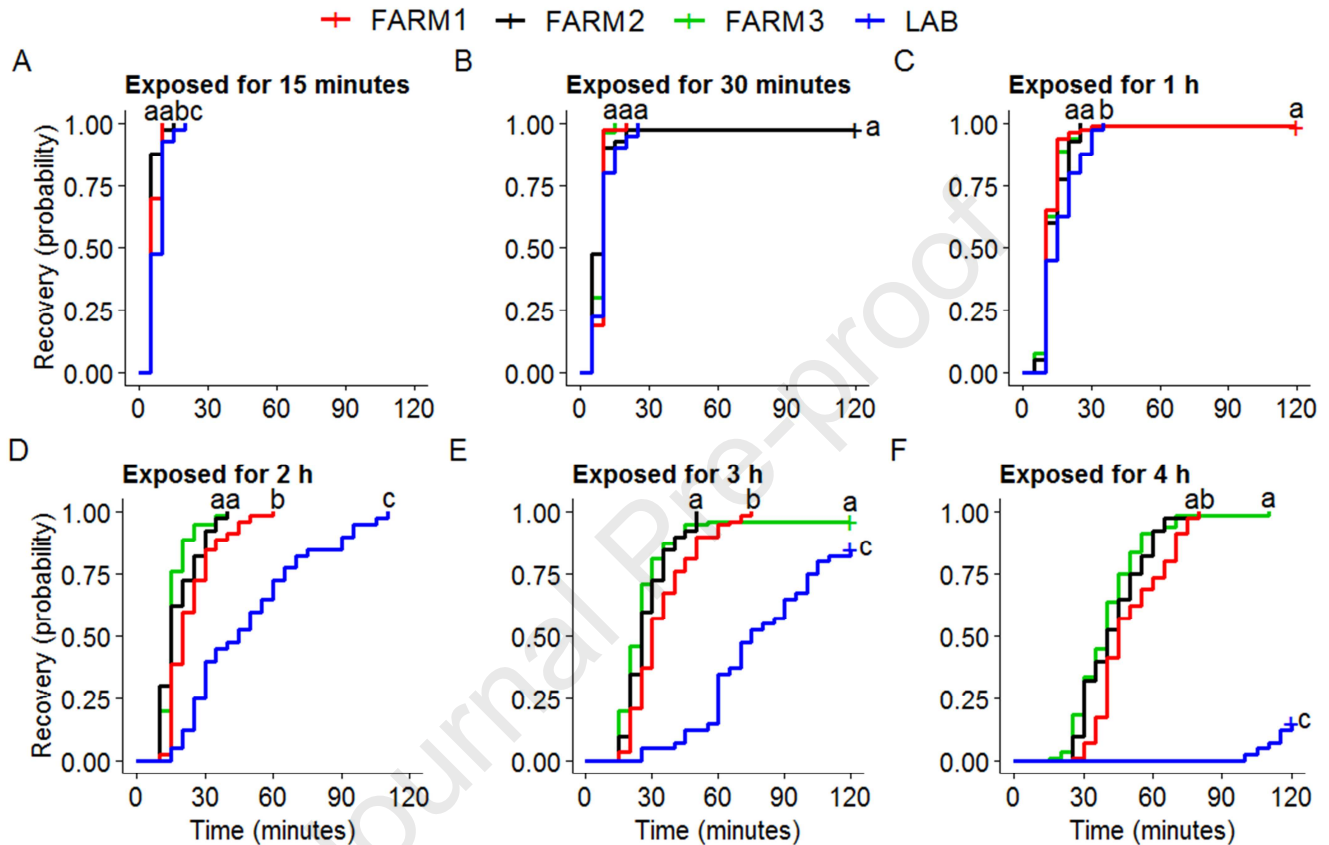

Figure 1. Recovery probability of individuals from four different populations of *A. diaperinus* exposed at 42.5 °C for six different durations: **A)** 0.25 hour (overall P < 0.001); **B)** 0.5 hour (overall P < 0.001); **C)** 1 hour (overall P < 0.001); **D)** 2 hours (overall P < 0.001); **E)** 3 hours (overall P < 0.001); **F)**

11

294 4 hours (overall P < 0.001). The red lines represent insects from FARM1, the black lines represent
295 the insects from FARM2, the green lines represent the insects from FARM3, and the blue lines
296 represent the insects from LAB. Different letters indicate differences among the recovery probability
297 curves within each duration.

298

299

300 **Figure 2.** Recovery probability of individuals from four different populations of *A. diaperinus*
301 exposed at -6 °C for six different durations: **A**) 0.25 hour (overall P < 0.001); **B**) 0.5 hour (P = 0.074);
302 **C**) 1 hour (overall P < 0.01); **D**) 2 hours (overall P < 0.001); **E**) 3 hours (overall P < 0.001); **F**) 4 hours
303 (overall P < 0.001). The red lines represent insects from FARM1, the black lines represent the insects
304 from FARM2, the green lines represent the insects from FARM3, and the blue lines represent the
305 insects from LAB. Different letters indicate differences among the recovery probability curves within
306 each exposure duration.

307

308 **3.2. Effect of daily heat spikes and cyfluthrin exposure**

309 The number of active lesser mealworms was significantly reduced when insects were exposed to
310 cyfluthrin at constant temperature (26 °C) as compared with control insects (P < 0.05 (95%
311 confidence level)) for all populations (Figure 3), except for FARM1 **(Figure 3, A; Supplementary File**

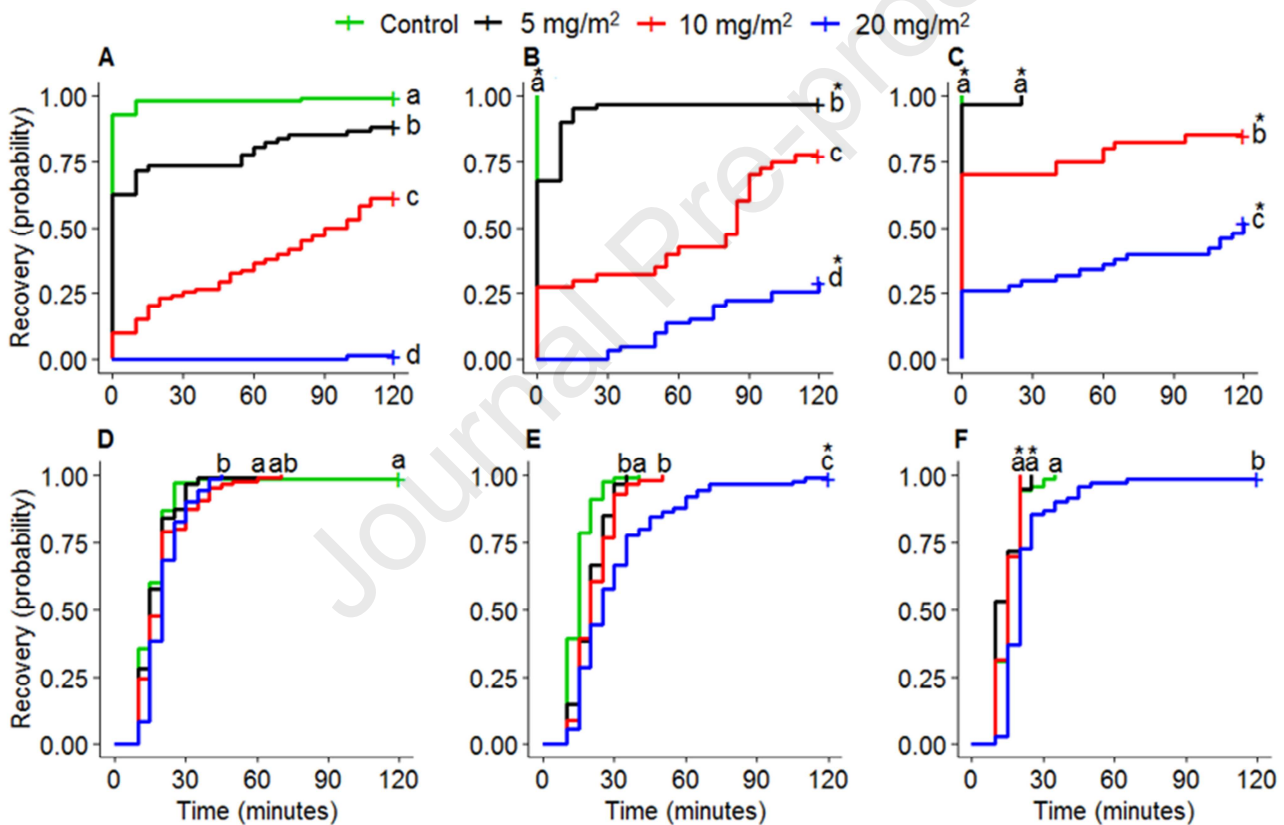312   **3)**. Sidak's multiple comparisons *post-hoc* tests following a two-way ANOVA showed that the

313   insecticide cyfluthrin and daily heat spikes (18h at 26 °C followed by 6h period at 38 °C for 8 days)

314   significantly decreased the proportion of active insects as compared with control and heat

315   treatment conditions (P < 0.05) for all populations **(Figure 3A-D; Supplementary File 3)**. Yet, the

316   proportion of insects that were scored as active remained close to 100% for FARM1. The proportion

317   of insects scored as active significantly differed among the "Cyfluthrin" and "Daily heat spikes &

318   Cyfluthrin" treatments for FARM2 (P < 0.05) and LAB (P < 0.05). There was no significant interaction

319   between daily heat spikes and cyfluthrin for any of the populations (P = 0.28 or higher) **(Figure 3, A-**

320   **D; Supplementary File 3)**.

321

322   **Figure 3.** Status of adult *A. diaperinus* from populations **A**) FARM1, **B**) FARM2, **C**) FARM3 after they
323   were exposed for eight days to control conditions (constant 26 °C), daily heat spikes (26 °C for 18h,
324   38 °C for 6h), cyfluthrin (20 mg/m^2) or daily heat spikes and cyfluthrin and **D**) LAB after eight days of
325   same exposure treatments, but with a cyfluthrin concentration of 0.5 mg/m^2. Different letters
326   indicate differences among the number of active insects within each population.

327

328   **3.3. Effect of daily heat spikes and cyfluthrin exposure on recovery from exposure to extreme**

329   **temperatures**

13

The recovery probability of adult *A. diaperinus* from FARM1 decreased with increasing doses of cyfluthrin after the insects were maintained for eight days under different temperature regimes and cyfluthrin concentrations prior to extreme temperature exposure (P < 0.001) **(Figure 4; Supplementary File 3)**. This was particularly evident for beetles exposed to heat (42.5 °C) **(Figure 4)**. Pre-exposure to a daily heat spike for eight days **(Figure 4, B-C)** accelerated the recovery of the insects after they were exposed at 42.5 °C for 2h, as compared with animals exposed at constant 26 °C **(Figure 4, A; Supplementary File 3)**. Conversely, the recovery curves remained comparable for insects that were exposed to extreme cold (-6 °C for 2h; **Figure 4, D-F)**, even though they were previously subjected to different heat spikes for 8 days.

Figure 4. Recovery probability of *A. diaperinus* from FARM1 observed every 5 minutes after a 2-hour exposure at 42.5 °C (A, B and C) or -6 °C (D, E and F). Before the test, the insects were exposed to different temperature regimes: (**A**) and (**D**) constant 26 °C; (**B**) and (**E**) 26 °C daily interrupted by 6h at 32 °C; (**C**) and (**F**) 26 °C daily interrupted by 6h at 38 °C. For each temperature regime, the insects were exposed to four different concentrations of the insecticide cyfluthrin: 0 (control), 5 (black lines), 10 (red lines) or 20 (blue lines) mg cyfluthrin/m^2. Stars (*) represent significant differences between the marked curve and the curve of the same colour drawn in Fig. 4A (constant temperature

347 regime used as the reference recovery probability). Different letters indicate differences among the
348 recovery probability curves within each temperature pre-treatment group.

349

350 **4. Discussion**

351 **4.1 Characterization of the thermal tolerance of the insects from different populations**

352 In this study, we were interested in comparing the basal thermal tolerance of adult *A. diaperinus*
353 from four populations through assessments of their ability to recover from different durations of
354 exposure to cold or heat. Adults from FARM1 and FARM2 were characterized by the greatest ability
355 to recover from thermal stress, in particular to heat exposure, while laboratory-reared adults
356 exhibited the lowest thermal tolerance, in line with our expectations. The thermal plasticity of lesser
357 mealworm populations is also consistent with the wide range of temperatures that can occur during
358 a poultry house production cycle. Acclimation or adaptation to thermally variable habitats may
359 widen the temperature tolerance range, as reported from other insect models (reviewed in Colinet
360 et al. 2015; Lachenicht et al. 2010). For instance, working with *Drosophila melanogaster* reared at
361 varying temperature regimes, Bozinovic et al. (2011) observed a reduction of the number of flies
362 entering into chill or heat coma when transferred to extreme temperatures, as compared with flies
363 reared at constant temperature. Overgaard, Hoffmann, and Kristensen (2011) evidenced that
364 acclimation of *D. melanogaster* to variable temperatures increased tolerance to both low and high
365 thermal stress. In addition, these authors showed that populations originating from temperate areas
366 exhibited a wider thermal tolerance than those sampled from more thermally stable tropical
367 environments. In sum, the lower thermal tolerance of adult *A. diaperinus* from LAB is consistent
368 with the available literature, and the hypothesis that poultry house-collected insects have higher
369 recovery probabilities than laboratory-bred individuals was confirmed.

370 In this work, insects from FARM3 exhibited a significantly lower thermal tolerance than their
371 counterparts sampled from the other two poultry farms. However, many other environmental
372 factors experienced by the insects in the poultry farm, such as pH and ammonia concentration in the
373 litter, or the presence and diversity of bacteria and viruses which trigger immune responses that can
374 alter the performance of other physiological traits, may have shaped the thermal tolerance of the
375 insects from poultry farms. Further, we can also expect the populations to be genetically distinct

376   from each other. As a result, variance in thermal tolerance among the insects from the three farm

377   populations is not surprising (Miller, Mohrenweiser, and Bell 2001).

378   **4.2. Effect of daily heat spikes and cyfluthrin exposure**

379   The previous experiment reported differences in the thermal tolerance among populations of *A.*

380   *diaperinus*. In particular, insects from FARM1 and FARM2 exhibited slightly higher capacity to

381   recover from heat exposure, suggesting that they would be better able to cope with daily heat

382   spikes, while insects from the LAB population should have had the lowest ability to deal with peaks

383   of heat. Yet, none of the populations were visibly affected by the daily heat spikes on their own.

384   Insects from FARM1 were capable of coping with the investigated dose of cyfluthrin at constant

385   temperature, while this condition was detrimental for the three other populations. As expected,

386   adults of *A. diaperinus* from LAB were more sensitive to the insecticide than those of the three

387   FARM populations. Differences in cyfluthrin resistance have already been reported in *A. diaperinus*

388   populations from Australia (Lambkin and Rice 2009), where resistance was generally related to the

389   extent and frequency of cyfluthrin applications experienced by the insects. Similarly, Chernaki-Leffer

390   et al. (2011) found strains of *A. diaperinus* of different pesticide (cypermethrin, dichlorvos and

391   triflumuron) susceptibility in Brazil, and Hamm et al. (2006) found strains of *A. diaperinus* with

392   different pesticide (cyfluthrin and tetrachlorvinphos) resistance in the United States. The latter

393   authors suggested that resistance alleles are present in the resistant populations of the lesser

394   mealworm, as may have been the case with our poultry house collected insects.

395   While daily heat spikes alone did not affect the recovery of the insects of the four tested

396   populations, the combination of insecticide and daily heat spikes significantly reduced the number

397   of active individuals in two (FARM2 and LAB) out of the four assayed populations. Warm

398   temperatures have been found to decrease the toxicity of the pyrethroids cypermethrin and

399   deltamethrin in the fly *Musca domestica* (Khan and Akram 2014), and in the Spotted bollworms

400   *Earias vittella* exposed to pyrethroids at higher temperature regimes (Satpute et al. 2007). Yet, these

401   authors used constant temperatures in their experiments. The increased negative effects of the

402   insecticide for adult *A. diaperinus* experiencing a daily heat spike of 6 hours is consistent with the

403   findings of several other studies (Delnat et al. 2019; Willming, Qin, and Maul 2013; Zhu, Sengonca,

404   and Liu 2006), who found increasing pesticide toxicity in insects subjected to daily bouts of high

405   temperatures. For instance, daily thermal fluctuations as low as 5 °C increased toxicity of

406   chlorpyrifos in damselfly larvae (Verheyen and Stoks 2019). The synergistic effects of thermal

407   variations and insecticides may occur under large thermal variations only, as reported in the vector

408   mosquitoes *Culex pipiens* (Delnat et al. 2019). The magnification of the effects of pyrethroids on *A.*

409   *diaperinus* we have found emphasizes the importance of considering daily thermal variations and

410   heat spikes in risk assessments of the sensitivity of biodiversity to pollution under different

411   scenarios of climate change, although a higher constant temperature equivalent of the average

412   temperature experienced by animals exposed to daily heat peaks would allow us to make stronger

413   conclusions on this point. At higher temperatures, pesticide uptake may be increased, as found by

414   Lydy, Belden, and Ternes (1999) and Buchwalter, Jenkins, and Curtis (2003). In parallel, elimination

415   rates of the toxicants, for example through temperature-dependent activation of enzymes, should

416   also be enhanced at warmer conditions. In sum, the available literature and our results could

417   indicate a promotion of insecticide resistant genotypes with increasing occurrence of extreme

418   weather events, which is consistent with documented links between climatic variables and evolution

419   of pesticide resistance in arthropod pests (Maino, Umina, and Hoffmann 2018). Facilitation of

420   insecticide resistance as a result of climate change could be a result of pleiotropy, shared genetic

421   mechanisms or genetic linkage (Pu, Wang, and Chung 2020).

422   **4.3. Effect of daily heat spikes and cyfluthrin exposure on recovery from exposure to extreme**

423   **temperatures**

424   Using adult *A. diaperinus* from FARM1, we tested how the sensitivity to extreme temperatures was

425   affected by thermal and insecticide pre-treatments. We expected higher proportion of recovery and

426   faster recovery from heat stress in insects exposed to a daily heat spike over eight consecutive days,

427   as extreme temperature tolerance is readily modified by a thermal conditioning processes, even in

428   the short-term (Chidawanyika and Terblanche 2010; Kingsolver et al. 2016; Weldon, Terblanche, and

429   Chown 2011). Our results are consistent with this expectation for heat-shocked insects. Daily peaks

430   at high temperatures may have acclimated the insects to heat, which does not necessarily prepare

431   them for cold exposures. Indeed, cold tolerance requires different mechanisms compared to heat

432   tolerance, by for instance increasing membrane fluidity instead of decreasing it (Neven 2000; Teets

433   and Denlinger 2013). This can explain why heat pre-exposures did not consistently affect cold

434   tolerance in this experiment. Similarly, it has been shown that acclimation to low temperature

435   increased cold tolerance, but did not affect heat tolerance in *Drosophila melanogaster* (Overgaard

436   et al. 2007).

437   For insects exposed to extreme heat, insecticide pre-treatment significantly reduced recovery

438   probability, both by generally increasing the recovery time and the number of beetles that did not

439   recover within the recovery period. In fact, beetles pre-exposed to the sublethal dose of 20 mg/m^2

440   at control temperature never recovered from the applied sublethal heat exposure. We also

441   observed that even a relatively low exposure dose (5 mg cyfluthrin/m²) had a significant effect on

442   the recovery probability of the individuals, thus providing supporting evidence for toxicant-induced

443   climate sensitivity (Hooper et al. 2013; Moe et al. 2013) in adult *A. diaperinus*. Several studies have

444   shown that an increase in temperature generally enhances negative effects of contaminants (Noyes

445   et al. 2009; Noyes and Lema 2015), but the number of studies that have tested the effects of

446   insecticide pre-treatments is rather limited as compared with investigations assessing the effects of

447   simultaneous exposures. Importantly, when working sequentially, i.e. by applying one stress before

448   the other, results can greatly vary depending on the specific chemical investigated (Matzrafi 2019).

449   Here, we can hypothesize that coma, which is related to nerve dysfunction, through decreased

450   membrane potential and reduced excitability of the neuromuscular system, occurs more quickly and

451   severely during heat treatment after cyfluthrin exposure, as cyfluthrin is an inhibitor of nerve cell

452   sodium channels. Meanwhile, further research is required to reveal the mechanistic processes

453   explaining the recovery patterns we observed here. For the insects exposed to extreme cold, the

454   effect was only observable after exposure to a dose that is close to the one recommended for use of

455   the insecticide in commercial products (20 mg cyfluthrin/ m²). A previous study did not find an effect

456   of pesticide exposure on freeze tolerance in an earthworm (Bindesbøl et al. 2009), which indicates

457   that cold tolerance is not as sensitive to insecticide exposure as heat exposure.

458   Our results also fit well with effects of pesticide exposure on temperature tolerance found in other

459   studies. For example, Op de Beeck, Verheyen, and Stoks (2017, 2018) found that damselfly larvae

460   that were sensitive to the pesticide chlorpyrifos also showed a reduction in heat tolerance after

461   exposure to the pesticide. The authors also showed that the reduction in heat tolerance was smaller

462   when the animals had been pre exposed to higher temperatures, a finding that is in line with our

463   study. In addition, Delnat et al. (2019) found that pesticide exposure decreased temperature

464   tolerance in mosquito larvae and males, and Meng, Delnat, and Stoks (2020) showed that pesticide

465 exposure prior to experiencing a heat spike reduced heat tolerance of mosquito larvae. This
466 reduction in temperature tolerance following pesticide exposure is also true for other taxa, as
467 critical thermal maximum was also reduced in fishes after exposure to endosulfan and chlorpyrifos
468 (Heath et al. 1994; Patra et al. 2007).

469 The effect of fluctuating temperature regime was also observable in animals who had been exposed
470 to insecticide prior to extreme temperature treatment. It is however difficult to determine whether
471 the observed increase in thermal tolerance after varying temperature regimes was caused by
472 increased tolerance to extreme heat, to insecticide exposure or a combination of the two (Oliver
473 and Brooke 2017). Further research into pesticide uptake and elimination in the animals in the
474 course of this experimental setup would be of interest to further elucidate the processes behind the
475 observed effect.

476 **5. Conclusion**

477 The present work reports that populations of the lesser mealworms have distinct abilities to recover
478 from thermal and insecticide stress, and their interactions. This finding emphasises the importance
479 of designing experiments including several populations when making assessments of the sensitivity
480 of a given species to environmental factors, and more particularly when these data nourish models
481 aiming at forecasting the future of biodiversity. No synergistic interaction was found when the lesser
482 mealworms were jointly subjected to daily heat spikes and cyfluthrin; yet, this type of experimental
483 procedure, which simulates the conditions that could be experienced by free-ranging organisms in
484 natural and anthropogenic environments, increase the ecological realism of the study. This is even
485 truer in a global warming context, as daily heat spikes are expected to become more frequent in
486 several habitats. Here, we also found the importance of working with field-collected insects. The
487 thermal tolerance and sensitivity to insecticide of the laboratory population was very different than
488 that of the field specimens, meaning that different conclusions would be drawn if working with the
489 former population only. Finally, and in line with earlier investigations, our study pointed out the
490 importance of considering the effects of multiple stressors, and the lasting effects they can have on
491 other traits of the insects.

492

493 **Acknowledgements**

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500   **Supplementary File 1**

501   To begin this study, we had to test the effects of different temperatures on the recovery capacity of

502   adult *Alphitobius diaperinus,* that is, the effects of exposure to extreme temperatures (cold, heat) on

503   the recovery ability and duration of the individuals.

504   Insects from the population FARM1 were used to describe the general temperature tolerance of the

505   species, and to find temperature exposures that would cause the adults to enter chill coma, yet

506   keeping the ability to recover (capacity to regain full movement) afterwards. Four to eight replicates

507   of 10 individuals in each container (test tube with rubber cap) were created for each exposure

508   temperature, starting at -4 °C for cold exposure, and at +42 °C for heat. At the end of the thermal

509   treatment, the insects were directly transferred to Petri dishes and observed every 5 minutes for 2h.

510   Each observation period lasted for up to 40 seconds, and individuals were registered as either

511   "Immobile, Impaired" or "Recovered". Animals were characterized as "Recovered" when they had

512   regained the ability to move normally. The temperature both in the cryothermostat and the room

513   where recovery observations took place was monitored using iButtons® (iButtonLink, LLC.,

514   Whitewater, USA).

515   Since thermal performance curves are often steep at high temperature extremes, and change less

516   quickly at cold temperature extremes (Huey and Stevenson 1979; Sinclair et al. 2016), we used small

517   increments of temperature in the heat exposure experiments (0.5 °C) and larger increments (2 °C)

518   for cold exposure. When having run three temperatures for a duration of 2 hours, we were able to

519   see the temperatures that would best describe the effects of different durations of exposure on the

520   thermal tolerance of adult *A. diaperinus* from different populations. These experimental conditions

521   were also used for comparing the effects of different daily heat spikes and insecticide exposures

522   prior to extreme temperature exposure.

523   In Appendix Figure 1, we see that the insects recovered very quickly from the least extreme

524   investigated temperatures; exposure to +42 °C did not even elicit heat coma from which the animals

525   could recover. Furthermore, the animals showed a low ability to recover from the most extreme

526   temperatures used: after an exposure to +43 °C, many insects were not able to recover within the

527   recovery period (Appendix Figure 1, A), and after 2 hours at -8 °C it took around 45 minutes before

528   half of the individuals had recovered (Appendix Figure 1, B).

529

530   Appendix Figure 1 illustrates the recovery time of adult *Alphitobius diaperinus* from FARM1 after
531   exposure to different extreme temperatures (represented by different coloured lines) for 2 hours. A)
532   Recovery curve of individuals exposed to extremely warm temperatures (42 to 43 °C) (overall P <
533   0.001). B) Recovery trajectory of individuals exposed to extremely cold (-4 to -8 *°C*) temperatures
534   (overall P < 0.001).

535   From these results, it can be concluded that adults of *A. diaperinus* have an extreme temperature

536   tolerance that is within the range that commonly characterizes insects (Addo-Bediako, Chown, and

537 Gaston 2000). We also see that the changes in recovery time with changing temperature follows a

538 pattern typical from thermal performance curves (Colinet et al. 2015; Huey and Stevenson 1979).

539 Specifically, great changes in thermal tolerance happens across a narrower temperature range at

540 extreme heat (here between 42 and 43 °C) compared to extreme cold (here -4 to -8 °C). We also

541 note that adults of *A. diaperinus* were stressed (went into chill coma), but all individuals recovered

542 from sudden exposure at 42.5 °C and -6 °C. Therefore, these conditions represent suitable

543 temperatures for comparing differences in recovery time among populations or treatment groups.

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546 **Supplementary File 2**

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Appendix figure 1 Schematic presentation of the design of experiment 1

Appendix figure 2 Schematic presentation of the design of experiment 2

Appendix figure 3 Schematic presentation of the design of experiment 3. The second part of the experiment (Exposure 2 and recovery observations) was identical to experiment 1, and can be seen in larger version in Appendix figure 1.

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565 **Supplementary File 3**

566 Appendix table 1: Detailed statistical results of experiment 1 (corresponding to results presented in

567 Figures 1 and 2 in the main text of the article). Pairwise comparisons (Log-Rank tests; graphs A-F)

568 were performed using the Survdiff function in R. Significant p values are presented in bold.

Recovery from extreme heat (42.5 °C)

Duration	15 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**4.90E-06**	-	-
FARM 1	**4.90E-06**	1.0000	-
LAB	**0.004**	**< 2.00E-16**	**< 2.00E-16**

Duration	30 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**0.0020**	-	-
FARM 1	**0.0020**	0.8152	-

Recovery from extreme cold (- 6 °C)

Duration	15 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.7078	-	-
FARM 1	**0.0105**	0.1059	-
LAB	**7.60E-06**	**0.0013**	**0.0082**

Duration	30 minutes exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.6470	-	-
FARM 1	0.1430	0.1430	-

	FARM 3	FARM 2	FARM 1
LAB	**0.0004**	**3.20E-14**	**< 2.00E-16**

	FARM 3	FARM 2	FARM 1
LAB	0.0720	0.1430	0.2050

Duration	1 hour exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**0.0001**	-	-
FARM 1	**4.90E-07**	0.9446	-
LAB	0.3298	**2.00E-08**	**2.70E-12**

Duration	1 hour exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.5049	-	-
FARM 1	0.8946	0.5049	-
LAB	**0.0039**	**0.0449**	**0.0080**

Duration	2 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**2.80E-06**	-	-
FARM 1	**2.30E-05**	0.2720	-
LAB	**0.0190**	**1.00E-07**	**1.70E-08**

Duration	2 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.3069	-	-
FARM 1	**1.80E-07**	**0.0042**	-
LAB	**< 2.00E-16**	**2.20E-11**	**1.60E-10**

Duration	3 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	**1.70E-08**	-	-
FARM 1	**3.40E-10**	0.6024	-
LAB	**0.0002**	**5.30E-14**	**1.50E-14**

Duration	3 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.4633	-	-
FARM 1	**0.0019**	**0.0080**	-
LAB	**2.00E-13**	**< 2.00E-16**	**< 2.00E-16**

Duration	4 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.0591	-	-
FARM 1	**5.00E-06**	0.0591	-
LAB	0.0121	0.0002	**1.10E-08**

Duration	4 hours exposure		
Population	FARM 3	FARM 2	FARM 1
FARM 2	0.4142	-	-
FARM 1	**0.0002**	**0.0137**	-
LAB	**< 2.00E-16**	**< 2.00E-16**	**< 2.00E-16**

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570 Appendix table 2: Detailed statistical results of experiment 2 (corresponding to results presented in

571 Figure 3 in the main text of the article). Overall ANOVA tests (type II) to the left, Sidak adjusted *post*

572 *hoc* test to the right. All data made using 95% confidence level, significant P-values are presented in

573 bold.

FARM1	Chisq	Df	P-value
Daily heat spikes	0.6414	1	0.4232
Cyfluthrin treatment	16.6845	1	**4.41E-05**
Daily heat spikes & Cyfluthrin	1.1667	1	0.2801

FARM1	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.931	0.973	a
Cyfluthrin treatment	0.947	0.983	ab
Control	0.968	0.994	b
Daily heat spikes	0.973	0.996	b

FARM2	Chisq	Df	P-value
Daily heat spikes	13.7	1	**0.000214**
Cyfluthrin treatment	973.44	1	**2.20E-16**
Daily heat spikes & Cyfluthrin	0.08	1	0.775383

FARM2	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.484	0.567	a
Cyfluthrin treatment	0.569	0.651	b
Control	0.981	0.999	c
Daily heat spikes	0	1	abc

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FARM3	Chisq	Df	P-value
Daily heat spikes	1.52	1	0.218
Cyfluthrin treatment	603.76	1	**2.00E-16**
Daily heat spikes & Cyfluthrin	0.02	1	0.8857

FARM3	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.329	0.469	a
Cyfluthrin treatment	0.377	0.519	a
Control	0.961	1	b
Daily heat spikes	0.961	1	b

LAB	Chisq	Df	P-value
Daily heat spikes	9.53	1	**0.002018**
Cyfluthrin treatment	631.06	1	**2.20E-16**
Daily heat spikes & Cyfluthrin	0.27	1	0.604759

LAB	LCL	UCL	Group
Daily heat spikes & Cyfluthrin	0.084	0.193	a
Cyfluthrin treatment	0.177	0.315	b
Control	0.929	0.999	c
Daily heat spikes	0.929	0.999	c

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582 Appendix table 3: Detailed statistical result of experiment 3 (corresponding to results presented in

583 Figure 4 in the main text of the article). Pairwise comparisons using Log-Rank test were performed

584 using the Survdiff function in R. Significant p values are presented in bold.

Extreme heat (42.5 °C) tolerance data

Insecticide conc.	Pre-treatment peaks:	non	
mg cyfluthrin/ m2	0	5	10
5	**1.60E-06**	-	-
10	**< 2.00E-16**	**3.40E-09**	-
20	**< 2.00E-16**	**< 2.00E-16**	**9.20E-14**

Insecticide conc.	Pre-treatment peaks:	32 °C	
mg cyfluthrin/ m2	0	5	10
5	**2.2E-06**	-	-
10	**2.0E-14**	**9.4E-09**	-

Extreme cold (- 6 °C) tolerance data

Insecticide conc.	Pre-treatment peaks:	non	
mg cyfluthrin/ m2	0	5	10
5	0.4598	-	-
10	0.0627	0.1072	-
20	**0.0064**	**0.0341**	0.7462

Insecticide conc.	Pre-treatment peaks:	32 °C	
mg cyfluthrin/ m2	0	5	10
5	**1.0E-05**	-	-
10	**3.6E-07**	0.25036	-

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20	**< 2.0E-16**	**< 2.0E-16**	**5.9E-07**

20	**5.2E-16**	**1.2E-05**	**0.00038**

Insecticide conc. mg cyfluthrin/ m2	Pre-treatment peaks:	38 °C	
	0	5	10
5	0.17	-	-
10	**2.7E-05**	**1.3E-05**	-
20	**1.4E-14**	**1.9E-15**	**7.7E-05**

Insecticide conc. mg cyfluthrin/ m2	Pre-treatment peaks:	38 °C	
	0	5	10
5	0.25	-	-
10	0.7	0.65	-
20	**5.0E-07**	**8.3E-08**	**1.3E-07**

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586 Appendix table 4: Bold numbers represent p values showing significant differences between graphs,

587 bold and italicized p values showing significantly slower recovery in insects having experienced daily

588 heat spikes in pre-treatment.

Pre-treatment	constant VS 32 °C peaks		constant VS 38 °C peaks	
Extreme temp.	42.5 °C	-6 °C	42.5 °C	-6 °C
Cyfluthrin conc. 0	**0.036**	0.092	**0.045**	0.29
Cyfluthrin conc. 5	**0.02**	0.07	**< 0.0001**	**0.0016**
Cyfluthrin conc. 10	0.068	0.62	**< 0.0001**	**0.00041**
Cyfluthrin conc. 20	**< 0.0001**	*< 0.0001*	**< 0.0001**	0.4

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863

Highlights:

- Thermal tolerance varied among populations of the lesser mealworm
- Detrimental effect of simultaneous exposure to stressors, but no interaction
- Pre-exposure to daily heat peaks decreased recovery time from heat shock
- Previous insecticide exposure reduced tolerance of extreme temperatures

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: