Sublethal larval exposure to imidacloprid impacts adult behaviour in *Drosophila melanogaster*

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Abstract

Pesticides are now chronically found in numerous ecosystems incurring widespread toxic effects on multiple organisms. For insects, the larvae are very exposed to pesticide pollution and the acute effect of insecticides on larvae has been characterised in a range of species. However, the carry-on effects in adults of sublethal exposure occurring in larvae is not well characterised. Here, we use a collection of strains of Drosophila melanogaster differing in their larval resistance to a commonly used insecticide, imidacloprid, and we test the effect of larval exposure on behavioural traits at the adult stage. Focusing on locomotor activity and on courtship and mating behaviour, we observed a significant carry-on effect of imidacloprid exposure. The heritability of activity traits measured in flies exposed to imidacloprid was higher than measured in controls and in these, courtship traits was genetically less correlated from mating success. Altogether, we did not observe a significant effect of the larval insecticide resistance status on adult behavioural traits, suggesting that selection for resistance in larvae does not involve repeatable behavioural changes in adults. This lack of correlation between larval resistance and adult behaviour also suggests that resistance at the larval stage does not necessarily result in increased behavioural resilience at a later life stage. These findings imply that selection for resistance in larvae as well as for behavioural resilience to sublethal exposure in adult will combine and impose a greater evolutionary constraint. Our conclusions further substantiate the need to encompass multiple trait measures and life stages in toxicological assays to properly assess the environmental impact of pesticides.

key words: locomotor activity, courtship, circadian rhythm, G-matrix, multitrait evolution

Introduction

The use of pesticides has steadily increased in the past 60 years so that pesticide molecules or their residues are now found globally and in every ecosystem (Vörösmarty et al., 2010; Roser & Ritchie, 2015; Cruzeiro et al., 2018). However, the long-term consequence of pesticide exposure at a reduced dose across different life-stages is rarely considered. In particular, an understanding of the carry-over effect of early exposure to a pesticide on later stages of the life cycle is limited to a few studies (Whitehorn et al., 2018; Müller et al., 2019)□.

Soon after insecticide application, the active molecules degrade and decay in concentration. Depending on the mode of application and the chemistry of these molecules, pesticides may also drift or wash away in effluent waters (Guedes et al., 2016; Carvalho, 2017)□. This implies that for a given treated area, a much greater area is exposed to a reduced concentration of pesticide, resulting in the exposure of a large population of arthropods that are not the target of the insecticide (Carvalho, 2017; Müller, 2018)□. While a decreased concentration of insecticide might not lead to dramatic levels of mortality, sublethal insecticide exposure has been inculpated with altering the physiology, behaviour and ultimately the population fitness of beneficial insects (Henry et al., 2012; Rundlöf et al., 2015; Cernansky, 2017)□. Thorough experimental testing of sublethal insecticide exposure has been conducted for multiple behavioural traits (Guedes et al., 2016)□, particularly the alteration of olfactory learning and memory in bees (Siviter et al., 2018)□. There has also been an emphasis on activity because of its importance on preying (Tooming et al., 2017)□ and foraging (Schneider et al., 2012; Lämsä et al., 2018)□.

The larval stage in insect generally corresponds to the longest-lasting, most actively feeding stage with relatively low mobility; it is thus the most likely likely to be highly exposed to pesticides with limited escape capacity. The adult stage corresponds to the reproductive stage with increased mobility and thus escape capacity (Chareonviriyaphap et al., 2013; Cordeiro et al., 2013; Nansen et al., 2016)□. These two stages have distinctive physiologies to complete differentiated functions; the effect of insecticide exposure may thus greatly differ across life stages (Zhu et al., 2002; Liu et al., 2013)□, and the consequences of an early exposure might amplify later in the life cycle (Tricoire-Leignel et al., 2012)□.

The central prediction in the emerging field of behavioural ecotoxicology is that exposure to neurotoxic agents would alter behavioural phenotypes, providing a biomonitoring tool 10 to 1,000 times more sensitive than mortality assays (Hellou, 2011; Peterson et al., 2017). Insect behaviour is a set of integrated physiological changes establishing the basis of strict stimulus–response associations (Wehner & Rössler, 2013). Studying the plasticity of insects' behavioural response to environmental challenge has raised considerable interest in entomology (Juliano et al., 2004; Liefting et al., 2009) and there is now an increasing focus on individual differences in behaviour that are repeatable across situations coined 'personality' (Tremmel & Müller, 2013; Kralj-Fišer & Schuett, 2014; Guedes et al., 2016)□. Difference in personality between genotypes is particularly relevant to support the persistence of genetic diversity within and among insect population, which was found to be a potential driver of behavioural resistance (Morales et al., 2013; Malia et al., 2016). Personality consolidation occurring when positive selection favours correlation between multiple behavioural traits promoting the emergence of discrete ‘behavioural syndromes’ which help support ecological functions (Chapman et al., 2011; Jandt et al., 2014). In the context of insects’ behavioural syndrome relying on multiple correlated traits, a corollary prediction is that exposure to toxic compounds would uncouple these traits, resulting in previously unobserved and potentially deleterious phenotype combinations.

Recurrent exposure of natural insect populations to an insecticide often results in the emergence and spread of resistance. However, the mechanisms of insecticide resistance have been occasionally shown to differ between larvae and adults (Liu et al., 2013; Bharati & Saha, 2018) and there is no consistent prediction of the effect of resistance mutations across these different life stages. General pleiotropic effects of resistance mutations have been shown to impact insect behaviour (Rostant et al., 2017; Somers et al., 2017), and these effects may be conserved across species (Foster et al., 2003). Pleiotropy for ecologically-relevant traits may induce non-independent response to selection and result in evolutionary constraints. Furthermore, there is an expectation that resistance mutations particularly those altering neurological function, carry a fitness cost (Kliot & Ghanim, 2012), but the experimental assessment of its evolutionary impact in natural populations remains a challenge (ffrench-Constant & Bass, 2017).

Locomotor activity is a fitness trait involved in a range of behaviours including stress response, foraging capacity and reproduction (Partridge et al., 1987; Feder et al., 2010; Overgaard et al., 2010; Bahrndorff et al., 2012). Locomotion can be measured quantitatively and has been shown to have high heritability in Drosophila melanogaster (van Dijken & Scharloo, 1979). Previous work in flies has also demonstrated that activity is highly sensitive to insecticide exposure (Denecke et al., 2015, 2017) and insecticide exposure was also shown to alter the response to the environment in adults (Fournier-Level et al., 2016). Because reproduction is the primary function of adults for non-social insects, courtship behaviour is a particularly relevant fitness trait in flies (Lasbleiz et al., 2006) that can bear immediate evolutionary consequence if perturbed. Alterations in the mating behaviour of resistant flies has been observed (Rostant et al., 2017; Somers et al., 2017), but surprisingly few studies have investigated the effect of larval pesticide exposure on flies’ courtship and mating.

Drosophila melanogaster has proved to be an excellent model to investigate the genetic response to chemical stress in insects. The Drosophila Genetic Reference Panel (DGRP) was established as a living library encompassing natural genetic variation segregating within population that can be phenotyped for a diverse array of evolutionarily-relevant traits (Mackay et al., 2012). This population has been assayed for resistance to a wide range of insecticides, both at the larval and adult stage (Battlay et al., 2016; Denecke et al., 2017; Schmidt et al., 2017; Duneau et al., 2018; Green et al., 2019)□□ and can be also used to infer the evolutionary consequences of insecticide use on a non-target insect (Robin et al., 2019)□. Among the insecticides that have been examined via the DGRP population is the neonicotinoid insecticide imidacloprid that alters neurotransmission by binding to nicotinic acetylcholine receptors in the central nervous system (Bai et al., 1991)□. Here, we ask if sublethal larval exposure to imidacloprid, a commonly used insecticide shown to have a large-scale evolutionary impact (Cressey, 2017; Fournier-Level et al., 2019)□, is conducive to an alteration of adult behaviour. Using a set of genetically diverse strains of D. melanogaster from the DGRP, locomotor activity in the presence and in absence of light cues and courtship and mating behaviour were monitored. The presence of a heritable behavioural response to imidacloprid exposure was tested under the hypothesis that 1) imidacloprid consistently alters the genetic components of the response and 2) imidacloprid alters the genetic correlation between behavioural traits. This was done to specifically assess the potential for imidacloprid to change the pattern of natural selection on behaviour in exposed populations and determine whether larval insecticide resistance correlated with particular adult behaviour.

Methods

Fly stocks and imidacloprid sublethal concentration determination

Data generated in Denecke et al. (2017)□ scoring the “Wiggle Index”, a larval activity index (Denecke et al., 2015)□ measured 0 and 120min after exposure to 25 and 100ppm of imidacloprid diluted in 5% w/v sucrose media, were used to select a restricted set of 16 genotypes from the original 178 genotypes characterised. These doses and times of exposure have been shown to discriminate resistant and susceptible genotypes in the DGRP population. The 16 genotypes were selected using Principal Components Analysis (PCA) as implemented in the R\FactoMineR package (Lê et al., 2008)□ to maximise the variance of the Wiggle index for the two time points and two doses (four conditions). These lines were: DGRP_88, DGRP_217, DGRP_235, DGRP_280, DGRP_301, DGRP_359, DGRP_386, DGRP_439, DGRP_486, DGRP_509, DGRP_513, DGRP_589, DGRP_705, DGRP_776, DGRP_802, DGRP_843. The 16 selected genotypes from the DGRP were originally sourced from the Bloomington Drosophila Stock Center. Fly stocks were maintained at 25°C under continuous light with a generation time of 2 weeks in 25mL vial containing ~10mL rich medium (maltose: 46g/L, dextrose: 75g/L, yeast: 35g/L, soy flour: 20g/L, maize meal: 73g/L, agar: 6g/L, acid mix: 14ml/L and tegosept: 16ml/L). The acid mix solution was made up of orthophosphoric acid (42ml/L), and propionic acid (412ml/L), while the tegosept solution was 50g tegosept dissolved in 950 ml of 95% EtOH.

To determine the concentration of imidacloprid corresponding to a sublethal exposure in larvae, for each of the 16 selected DGRP genotypes, a mix of 25 first or second instar larvae were placed in 25mL vials containing ~10mL rich medium supplemented with either 0 (control), 0.04, 0.08, 0.12 or 0.2ppm of imidacloprid (Confidor commercial formulation containing N-Methyl-2-pyrrolidone as solvent, Bayer), each concentration and genotype was repeated 4 times. Survival was counted on day 15 as the number of emerged adults present in the tube. The significance of the difference in survival of flies per vial between the vials containing imidacloprid and the control was tested using a t-test in order to determine the maximum No Observable Effect Concentration which was subsequently used as sublethal concentration. The flies used in the behavioural experiments were 3 to 7 days old and bred using the same procedure, except ~50 first and second instar larvae (instead of exactly 25) were transferred in each vial containing the sublethal concentration of imidacloprid.

Locomotor activity

Fly activity was measured using the Drosophila Activity Monitoring system (DAM6, Trikinetics, Waltham, MA, USA). The DAM6 system consists of an acrylic support where tubes carrying flies are inserted so that infrared beams record the sum of all crossing of the tube for the ten flies over time as a proxy for activity. Each 25mmx125mm tube containing 10 flies of a single sex was inserted in the DAM6, and the flies were allowed to recover from manipulation stress for an hour after which the number of crossings of the infra-red beams was recorded every two minutes over 6 days with 12h light/12h dark for the first 3 days and continuous darkness for the next 3 days. This was done to test the effect of insecticide on circadian rhythm with or without entrainment as a key neuro-physiological trait. The persistence of circadian rhythm was shown to be adaptive in insects both to maintain physiological robustness despite day-to-day variation but also to modulate learning and memory (Decker et al., 2007)□. The insecticide exposure treatment compared flies which were exposed to sublethal concentrations of imidacloprid as larvae (from 1st or 2nd instar until adult emergence) to flies reared on rich media. The sex treatment independently compared males and females. Each treatment per sex per genotype combination was repeated 12 times for exposed flies

$$y = C + \sum_{i=1}^{2} sin(2i\pi/\tau * t) + cos(2i\pi/\tau * t)$$

tes as a proxy for locomotor activity, activity-based behavioural traits were computed as described in Table 1. The circadian parameters (baseline activity, activity amplitude, period and phase) were estimated in a two-step method by first estimating the period τ using a Fourier transform-based spectral density estimation of the time series data and then estimating the other parameters by fitting a linear model to a second-harmonic sine cosine function of the time series as : (eqn. 1), where y is the locomotor activity, C is the baseline activity or model intercept and t is the time in minutes. The phase was estimated as: and then the amplitude as: , where βs are the regression coefficients corresponding to the effects measured in eqn. 1.

Courtship

Courtship assays were performed using pairs of flies exposed or not to imidacloprid using the same protocol as for the activity assay except that female flies were collected within 12 hours post adult emergence to make sure they had not mated. Sterile 6-well culture plates were used as courtship arenas that were filmed from above on a white background for ten minutes. Each treatment (four orthogonal combinations of imidacloprid-exposed or control and male or female) per genotype combination was repeated four to six times. Courtship steps were scored on the video capture based on the courtship steps described in Sokolowski (2001); the tapping step was discarded as it was too ambiguous and the orientating, singing, licking, attempted copulation and copulation were scored. To aid the detection of courtship steps, a suite of custom scripts (https://github.com/aflevel/FlyCourtship) using computer vision was developed to identify times of decreased movement corresponding to potentially interesting times that were then manually inspected. For each video, time to the first occurrence of a courtship step, if present, was recorded.

Statistical analyses

Statistical analyses were conducted using the R statistical software. (Lê et al., 2008). Single trait analyses were performed using general linear mixed-models implemented in the R\lme4 package (Bates et al., 2015) using sublethal insecticide exposure and sex as fixed effects and genotypes as a random effect; best models were selected based on Akaike Information Criteria (AIC). Broad-sense heritabilities were calculated for each trait using the clonal design by extracting the variance component due to the genotypes effect divided by the total trait variance. To confirm the result obtained for the locomotor activity analysis, a Monte Carlo simulation procedure was established by simulating 1000 times a set of 18 quantitative traits with their heritabilities equal to the broad-sense heritabilities estimated for the observed traits in absence or presence of imidacloprid with a sample size equal to the one of the experimental design. Pairwise t-tests were then performed between the 1000 sets of 18 simulated heritabilities and the estimated heritabilities and the t-statistics recorded. We finally compared the t-statistic obtained from the pairwise t-test on observed data to the 95-percentile of the distribution of the t-statistics obtained through simulation.

Multitrait analyses were performed using the R\MCMCglmm package (Hadfield, 2010), building a different model for each insecticide exposure treatment which distinguished female and male exposure. For the locomotor activity analysis, traits were modelled as normally distributed using sex as a fixed effect; for the courtship analysis, only likelihoods of completion of each step were modelled as logistically distributed. The Markov Chain Monte-Carlo procedure was carried out using 20 independent replications of 1,100,000 iterations with 100,000 burn-ins and a thinning period of 10,000. The estimates of the parameters (genetic variance and covariance) were obtained as the mean of the posterior distribution averaged across the 20 replicates.

Results

Larval response to imidacloprid and determination of sublethal exposure

The 16 DGRP lines selected were exposed to four concentrations of imidacloprid, and only 0.2ppm, the highest concentration tested, was found to have a significant impact on insect mortality (GLM, $|t|_{63}$=2.41, P=0.0356, Fig. 1a). To set the sublethal larval exposure to be subsequently used throughout the behavioural experiments, we determined the highest imidacloprid concentration in the food that did not significantly increase mortality compared to control. Of the four concentrations tested, 0.12ppm was the highest dose tested that did not significantly increased mortality (GLM, $|t|_{63}$=1.67, P=0.2096) and also retained a high level of variation in mortality within the population. The best linear unbiased predictor of the mortality response to 0.2ppm of imidacloprid for each genotype was used to determine the resistance status of these genotypes (Fig. 1b).

Sublethal effect of imidacloprid on activity and circadian entrainment

Exposure to imidacloprid at the larval stage had a significant effect on seven out of 16 behavioural traits measured through locomotor activity monitoring (highly active time, time asleep, total activity, baseline activity with light entrainment, activity amplitude with light entrainment, circadian period in the dark and activity in the last hour of light; GLM, $|t|_{218}$>2.13, P<0.034; Fig. 2 and Table 1). All significant effects were negative showing a reduction in activity in adults consecutive to larval exposure to imidacloprid (Table 1). Out of the 16 behavioural traits, only three (inactive time, inactive time change and persistence of circadian rhythm) did not show a difference between sexes (GLM, $|t|_{218}$>2.41, P<0.016). We detected no significant genotype-by-exposure interaction for the 16 traits tested, suggesting that despite extensive genetic variation in response to larval exposure, the adult response to imidacloprid was similar across genotypes. Therefore, the imidacloprid larval resistance status determined through the mortality assay did not consistently affect the adult behavioural traits analysed.

The complexity of the behavioural response was analysed using PCA. The first two principle components (PCs) explained together 46% of the variance in the data (Fig. 3a-b) and nine PCs were required to explain 90% of the variance, highlighting the high complexity of the data. Both imidacloprid exposure and sex had a significant effect on the variation along the first principal component (GLM, for imidacloprid exposure: t_{218}=-2.59 and P=0.014; for male sex: t_{218}=5.17 and P=5.28x10^{-7}). Total activity and time inactive were the two traits contributing the most to the first PC.

The high correlation between traits motivated the use of multi-trait linear mixed-models to estimate the genetic variances and covariances for the traits, computing separate models for imidacloprid-exposed flies and controls (Fig. 3c). Broad-sense heritabilities were high for all traits, ranging from 0.54 to 0.92 for the flies exposed to imidacloprid and from 0.46 to 0.93 for the control (Table 1). Interestingly, the exposure to imidacloprid at the larval stage increased heritability by 4.1% in average (paired t-test, t_{16}=-3.00, P=0.0090). We tested through simulation if this could be due to the unbalanced number of replications between imidacloprid exposed (n=12) and control flies (n=4) for each sex and genotype and found that in 95% of the simulations for the difference was less than 4.1%, suggesting this result is significant with a 5% risk. Matrices of genetic variance and covariance (G-matrices) extracted from the models were of full-rank, showed no difference in structure (Mantel test, r=0.99, P=0.001) and had equal effective dimension of 1. However, six out of 16 traits showed significant change in their correlation with the other traits: inactive time, highly active time, amplitude and phase of circadian activity under light entrainment, phase of circadian rhythm in the dark and persistence of circadian rhythm (paired t-test, $|t|_{16}$>2.18, P<0.05, Fig. 3c). This indicates that imidacloprid larval exposure did affect the genetic component of locomotory and activity behaviour in adult which is likely to have an evolutionary significance.

Alteration of mating behaviour

Exposure to imidacloprid at the larval stage affected all the five courtship traits measured, either in terms of likelihood of completion or time to completion of a given step (Table 2). Only for likelihood of orientation and time to the first copulation attempt did we not observe an effect of insecticide exposure. The imidacloprid treatment primarily affected females and we did not observe traits where taking into account the specific exposure of the male improved the model. The effect of sex-specific imidacloprid exposure was small except on the likelihood of licking and attempted copulation but nonetheless improved the model fit to the data based on AIC compared to the alternative null hypothesis. However, likelihood of copulation success was affected by both female and male exposure together with their interaction. Courtship traits showed a substantial genetic basis except for time to first attempted copulation with an estimated heritability ranging from 0.18 to 0.4. These estimates were considerably smaller than those of activity traits, and likelihood and time to copulation traits had their lower 2.5%-confidence interval overlapping with 0. Furthermore, including a genotype-by-exposure effect never improved the fit of the model for any tested trait, signifying that the effect of the sex-specific exposure was similar across lines, irrespective of the larval resistance status. These data suggest that resistance level of a genotype did not impact the effects of sublethal larval exposure on adult behaviour.

The courtship steps were followed in their expected order in 335 out of 349 assays with only 11 assays where a step was skipped (excluding attempted copulation, Fig. 4). Interestingly, all 3 assays where step order was inverted corresponded to conditions where both female and male had been exposed to imidacloprid. Male exposure increased blocking courtship at the singing step while female exposure increased blocking at the licking step. Strikingly, we found that exposing only one of the two sexes to imidacloprid led to a higher copulation success (12%) than when both or neither flies were exposed (3.5%, Fig. 4). To provide statistical support to these findings, we analysed the genetic variance and covariance between likelihood of completion of the courtship steps in a multi-trait model. This was not done for the time to completion data which is only measurable when the corresponding step is successfully completed. The genetic variance and covariance were also of full-rank, but this time significantly different between the control where neither female or male were exposed to imidacloprid and the treatment where either female or male or both were exposed (Mantel test, r=0.79-0.86, P>0.058, Fig. 5). We found no statistical differences among the insecticide treatments (Mantel test, r=0.89-0.95, P<0.025). The likelihood of each courtship step occurring was highly correlated in the controls, reflecting the expected importance of each step towards copulation success. In the contrary, exposure of female to imidacloprid induced a negative correlation between the three first steps of the courtship and final copulatory success; this pattern was accentuated to the attempted copulation when the male was also exposed. The courtship analysis underscores the paradoxical result that female exposure decreased the importance of the preliminary steps allowing for greater copulation success while male exposure is more generally deleterious to copulation success.

Discussion

Adult behaviour is affected by larval insecticide exposure

A sublethal exposure to imidacloprid at the larval stage was shown to impact Drosophila adult locomotor activity and courtship routines. Our results largely corroborate the findings from other studies that imidacloprid exposure reduces activity and therefore has potential effects on foraging, reproduction and long term survival (Haynes, 1988; Desneux et al., 2007; Müller, 2018) as primarily investigated in bees (Rundlöf et al., 2015; Wu-Smart & Spivak, 2016; Lämsä et al., 2018). On rare occasions, the effect of sublethal insecticide exposure has also been associated with a burst of activity as for a pyrethroid insecticide in the carabid beetle Platynus assimilis (Tooming et al., 2017) which implies that escape could be a mechanism of behavioural resistance (Bezzar-Bendjazia et al., 2017; Müller, 2018). A similar burst of activity was reported for Aedes aegyptii pupae exposed to imidacloprid (Tomé et al., 2014)□.

The effect of insecticides on mating behaviour has generally been shown to be detrimental (Tappert et al., 2017)□. owing to the "info-disruptor" effect of insecticide residues (Tricoire-Leignel et al., 2012)□ on pheromone communications as demonstrated in both pest (Evenden et al., 2005; Navarro-Roldán & Gemeno, 2017)□ and beneficial arthropods (Wang et al., 2018)□. A few notable exceptions have nonetheless been reported: in the pest moth Agrotis ipsilon it was shown that a neonicotinoid insecticide enhanced the response to sex pheromones (Rabhi et al., 2014)□. Interestingly, an experience-dependent response was also detected with re-exposure leading to increased effect on pheromone sensitivity as measured through male orientation response assay (Abrieux et al., 2016)□. A similar impact on male reproductive behaviour was found for imidacloprid where a sublethal exposure of the neotropical brown stink bug Euschistus heros increased male reproductive fitness (Haddi et al., 2016)□.

Most tests performed using sublethal concentrations of insecticides measured the immediate response to acute exposure (Müller, 2018)□. In contrast, our experimental design that exposed larvae and tested adults has not been widely employed and brings new insights that are relevant in an eco-evolutionary context. This type of design involving early exposure to low insecticide dose has been particularly useful for the empirical exploration of hormesis (Guedes & Cutler, 2014)□ and can be advantageously used in the context of longitudinal insecticide assays testing life-long effect. Even if no molecular mechanism is known to be affected by insecticide exposure over the transition from larvae to adult in insect, expression changes in the transcriptome of adult bees exposed to imidacloprid at the larval stage has been documented (Wu et al., 2017)□. Phenotypic effects of early exposure have been documented for the exposure of Pieris brassicae larvae to imidacloprid in concentration very similar to our experimental conditions and was shown to decrease adult body size (Whitehorn et al., 2018)□. Our study clearly demonstrates the significance of life-long effects of sublethal insecticide exposure which will need to be further assessed for other ecologically-relevant traits and taxa.

Sublethal exposure could impact the evolution of behavioural traits

The sublethal exposure to imidacloprid will be of evolutionary relevance in the presence of heritable variation for the traits affected that segregates in natural populations. We have measured significant heritabilities for all locomotor activity traits as well for most courtship traits (Table 2). Significant albeit lower levels of heritability had been estimated in the DGRP using a larger collection of genotypes and measuring similar circadian locomotor activity (Harbison et al., 2019)□ and courtship traits (Gaertner et al., 2015)□. The DGRP represents relatively recently sampled natural genetic variation from a single population and, with the caveat that the flies are highly inbred, these findings are expected to be relevant to other natural insect populations. We showed that levels of heritability increased significantly when flies were exposed to insecticide, which suggests that selection for behavioural traits with imidacloprid is expected to be more efficient at purging maladapted variants and conducive to greater loss of genetic diversity. If evolutionary theory suggests that heritability of a trait under directional selection is increased by stressful conditions (Hoffmann & Merilä, 1999)□, there is limited empirical support for a generalisation of this phenomenon (Rowiński & Rogell, 2017)□. Such a pattern was however previously observed in natural populations of D. melanogaster historically selected for DDT resistance (Fournier-Level et al., 2016)□ where the increased heritability for locomotor traits gradually decreased as natural selection favoured resistance.

Importantly, we detected no consistent effect of the level of larval resistance on adult behaviour when the set of genotypes was specifically selected to capture a maximum of genetic diversity for larval resistance. The correlation of larval resistance with adult resistance had been previously studied and yielded mixed conclusions. There have been enough instances where no correlation (Arnold & Whitten, 1975; Liu et al., 2013; Bisset et al., 2014)□ or inconsistent correlation was found across insecticides for a given species (Brewer et al., 1990)□ to raise the question of whether specific resistance mechanisms provide a life-long effect. In honey bees, it was further demonstrated that sensitivity to imidacloprid changed dramatically with age and genetic background (Rinkevich et al., 2015)□ which brings the possibility of physiological and genetic differences in resistance across life stages. Our study similarly supports life-stage differences in the effect of insecticide resistance as resistant larvae showed the same adult behavioural response as susceptible ones. In these cases, multiple traits and underlying loci responding to selection for the same insecticide pressure at different life stages could lead to a complex pattern of evolution.

Will imidacloprid exposure disrupt the correlation of behavioural traits?

The multifaceted nature of the response to sublethal insecticide exposure and the pleiotropic nature of resistance mutations, whether these are costly or not, are likely to result in more intense and multivariate response to selection than previously appreciated. However, theoretical perspectives on multivariate evolution supported by empirical findings point to the fact that despite abundant genetic variation, traits are genetically correlated and biological systems evolve in a narrow trait space (Kirkpatrick, 2009; Walsh & Blows, 2009). This means that selection on individual trait produces a limited and often constrained response. This is true of behavioural traits that are often correlated to define “behavioural syndromes” and a meta-analysis of behavioural syndromes including data from Drosophila found that traits underlying these were under strong evolutionary constraint (Dochtermann & Dingemanse, 2013)□.

The concept of personality has been used in insects to describe an integrated and persistent set of behavioural syndromes defined from multiple empirical trait measures (Morales et al., 2013)□. Alternative behavioural syndromes consolidate into distinct ‘personalities’ to promote specific ecological strategies, as for example early and fast versus late and slow life-cycling (Tremmel & Müller, 2013)□. Furthermore, environmental stress at early life stages was shown to impact the expression of personalities in insects (Tremmel & Müller, 2013)□. Our test on principle components allowed to capture personality based on multiple traits from the activity data and did show an effect of sex and imidacloprid exposure but not larval resistance status. This is comparable to a study on a weevil species testing the effect of resistance to deltamethrin on insect personalities which also found no impact of insect resistance (Morales et al., 2013)□. Nonetheless, our analysis suggests imidacloprid increases heritability, which means behavioural syndromes become more repeatable and personality more differentiated among genotypes upon insecticide exposure. This observation raised the possibility that in the presence of highly differentiated personalities, a brutal selective pressure exerted on a single trait could render a complete ecological strategy maladaptive; the aggravated evolutionary response would be due to negative selection for specific trait values associated with this strategy through personality consolidation.

We have observed particularly marked sex differences, and our study points to the fact that insecticide exposure increased these sex differences. This underscores the potential for insecticide exposure to increase the asymmetry of the evolutionary response across sexes under chronic but yet sublethal exposure. This was already identified as the potential source of intralocus sexual conflict in a natural population of D. melanogaster where DDT resistance mediated by the Cyp6g1 bears a cost only in female (Hawkes et al., 2016). An asymmetric effect of exposure on courtship also showed a strong context-dependence in weevil where despite bearing no apparent cost, deltamethrin resistance did lead to strong sexual selection against resistant strains (Guedes et al., 2017)□. Altogether our analysis supports the fact that sublethal insecticide exposure substantially impacts behaviour, in a way that extends beyond its adverse physiological effect, and may have under-appreciated evolutionary consequences on species and biodiversity. Insects are particularly exposed to environmental stresses living in increasingly polluted ecosystems and facing the impact of climate change. Their life-history and development are tightly dependent on the correct integration of external cues and it becomes increasingly clear that exposure to even small concentrations of pesticides can have very adverse consequences.

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Tables:

Table 1: Effect of imidacloprid exposure on locomotor activity traits.

Activity trait	Description	unit	gender effect (P-value)	imidacloprid effect (P-value)	Heritability controls (95% CI)	Heritability with imidacloprid (95%CI)
total activity	sum of all infra-red beam crosses over the full experiment	number of crosses over 7 days	6214.76 (P=1.86e-05)	-3857.47 (P=1.77e-02)	0.86 (0.74-0.93)	0.88 (0.79-0.94)
highly active time	time spent with a level of activity greater than the median level acivity	time (2 minutes resolution)	0.18 (P=2.41e-05)	0.1 (P=2.84e-02)	0.61 (0.42-0.79)	0.65 (0.5-0.8)
activity peaks	number of times the derivative of the smoothed activity reached a maximum	number of peaks over 7 days	1.41 (P=1.41e-15)	-0.12 (P=5.09e-01)	0.93 (0.87-0.97)	0.93 (0.88-0.97)
inactive time	time spent with no activity	time (2 minutes resolution)	-0.04 (P=8.60e-02)	0.06 (P=3.38e-02)	0.72 (0.53-0.88)	0.8 (0.66-0.91)
inactive time change	difference in the time spent inactive for flies during the 24h dark period and the 12h light/12h dark entrainment period	ratio	-0.09 (P=1.10e-01)	-0.07 (P=2.70e-01)	0.62 (0.42-0.79)	0.7 (0.53-0.84)
morning activity	activity during the first hour of light for the 12h light/12h dark entrainment period	number of crosses over 1 hour	364.46 (P=7.26e-10)	-111.89 (P=8.23e-02)	0.74 (0.57-0.86)	0.71 (0.56-0.83)
evening activity	activity during the last hour of light for the 12h light/12h dark entrainment period	number of crosses over 1 hour	499.95 (P=3.59e-13)	-152.21 (P=3.84e-02)	0.8 (0.64-0.9)	0.84 (0.74-0.92)
baseline activity (light)	intercept of the 2-harmonic cosine circadian model fitted to the 12h light/12h dark entrainment period	number of crosses over 2 minutes	1.19 (P=2.56e-04)	-0.97 (P=8.70e-03)	0.82 (0.69-0.91)	0.83 (0.73-0.91)
activity amplitude (light)	coefficient for the cosine term of the 2-harmonic cosine circadian model fitted to the 12h light/12h dark entrainment period	number of crosses over 2 minutes	0.9 (P=1.68e-02)	-1.12 (P=8.64e-03)	0.64 (0.47-0.81)	0.57 (0.43-0.74)
phase (light)	phase of the circadian rhythm for the 12h light/12h dark entrainment period ie. delay between the first hour of light and the maximum activity	time (2 minutes resolution)	37.29 (P=2.95e-03)	-22.61 (P=1.10e-01)	0.52 (0.35-0.69)	0.56 (0.41-0.72)
period (light)	periodicity of the circadian rhythm for the 12h light/12h dark entrainment period	time (2 minutes resolution)	-153.73 (P=5.84e-06)	-8.55 (P=8.20e-01)	0.81 (0.67-0.91)	0.86 (0.78-0.93)
baseline activity (dark)	intercept of the 2-harmonic cosine circadian model fitted to the 24h dark period	number of crosses over 2 minutes	1.32 (P=2.65e-05)	-0.64 (P=6.60e-02)	0.82 (0.67-0.91)	0.83 (0.72-0.91)
activity amplitude (dark)	coefficient for the cosine term of the 2-harmonic cosine circadian model fitted to the 24h dark period	number of crosses over 2 minutes	1.39 (P=1.82e-08)	-0.35 (P=1.97e-01)	0.67 (0.48-0.82)	0.73 (0.59-0.85)
phase (dark)	phase of the circadian rhythm for the 24h dark period ie. delay between the first hour of light and the maximum activity	time (2 minutes resolution)	84.67 (P=2.32e-14)	-14.75 (P=2.09e-01)	0.46 (0.3-0.65)	0.6 (0.47-0.76)
period (dark)	periodicity of the circadian rhythm for the 24h dark period	time (2 minutes resolution)	-136.85 (P=8.58e-05)	-83.58 (P=3.27e-02)	0.77 (0.6-0.88)	0.91 (0.84-0.95)
persistence of circadian rhythm	Goodness-of-fit of the circadian model for the 12h light/12h dark entrainment period fitted to the 24h dark period data	Proportion (R^2)	-0.02 (P=3.88e-01)	0 (P=8.43e-01)	0.81 (0.5-0.93)	0.86 (0.58-0.95)

Table 2: Effect of sex-specific imidacloprid exposure on courtship and mating traits

			Sex-sepcific imidacloprid exposure		
Courtship step	Description	Unit	female effect	female x male effect	Heritability (95% CI)
Orienting	Position of the male heading toward the female	presence/ absence	NI	NI	0.4 (0.13-1)
		time to first occurrence	0.58 (P=1.88e-02)	NI	0.4 (0.13-1)
Singing	Male vibrating a single wing	presence/ absence	0.45 (P=6.29e-02)	NI	0.36 (0.11-1.04)
		time to first occurrence	-21.17 (P=2.39e-01)	NI	0.26 (0.1-0.6)
Licking	Male labrum contacting female genitalia	presence/ absence	0.82 (P=5.55e-04)	NI	0.34 (0.11-0.96)
		time to first occurrence	-21.88 (P=3.91e-01)	NI	0.18 (0.03-0.49)
Attempted copulation		presence/ absence	0.64 (P=8.44e-03)	NI	0.32 (0.09-0.96)
		time to first occurrence	NI	NI	0.01 (0-0.17)
Copulation		presence/ absence	1.56 (P=2.04e-02)	1.21 (P=8.56e-02)	0.31 (0-1)
		time to first occurrence	47.35 (P=2.82e-01)	NI	0.31 (0-1)

NI: not included when not improving the fit of the model based on AIC

Figure legends

Figure 1: a) Effect of imidacloprid concentration on the proportion of flies emerged. The proportion of flies emerged after 15 days as a proxy for mortality was used to determine the highest imidacloprid concentration (0.12ppm) that did not increase mortality with respect to control (0ppm). Four replicates of each of the 16 genotypes were used (n=64 for each dose). The thick line represents the population mean, the box the interquartile distance and the whisker the 95%-ile distance. **b) Proportion of flies emerged when exposed to 0.2ppm of imidacloprid for the 16 DGRP genotypes tested.** Best linear unbiased predictors of the response was used as a metric of the resistance status of each genotype (n=4 for each genotype) . Fly genotypes showing a proportion of emergence 20% greater than the population average were classified as resistant (upper dashed line, turquoise); fly genotypes showing a proportion of emergence 20% lower than the population average were classified as susceptible (lower dashed line, red); others were classified as intermediate.

Figure 2: Example of locomotor activity profile over time (dark grey) **for DGRP_386.** White boxes represent light period, light grey boxes represent dark phase, the dashed black line mark 24h periods and the red line represent the experimental transition between light entrainment and

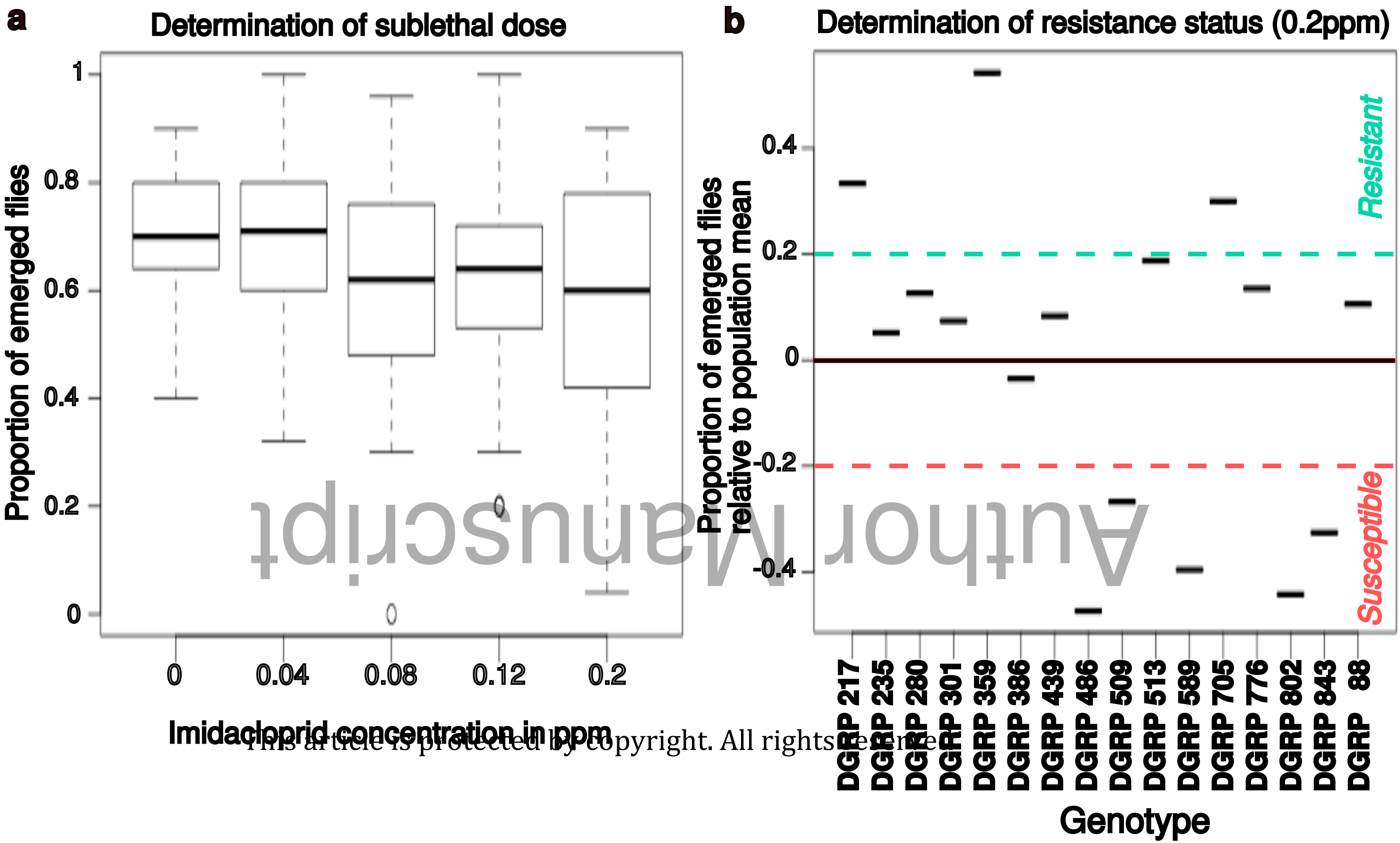
complete darkness. The solid orange lines represent the modelled activity during the entrainment phase, the solid blue lines represent the modelled activity during the complete darkness phase and the dashed orange lines represent the modelled activity during the entrainment phase projected onto the complete darkness phase. The top left-panel shows an example of profile for female control (CT) with the annotated phase (Φ), period (τ), amplitude (A) and baseline activity (C), the bottom-left a female imidacloprid-exposed (IMI), the top-right a male control and the bottom-right a male imidacloprid-exposed.

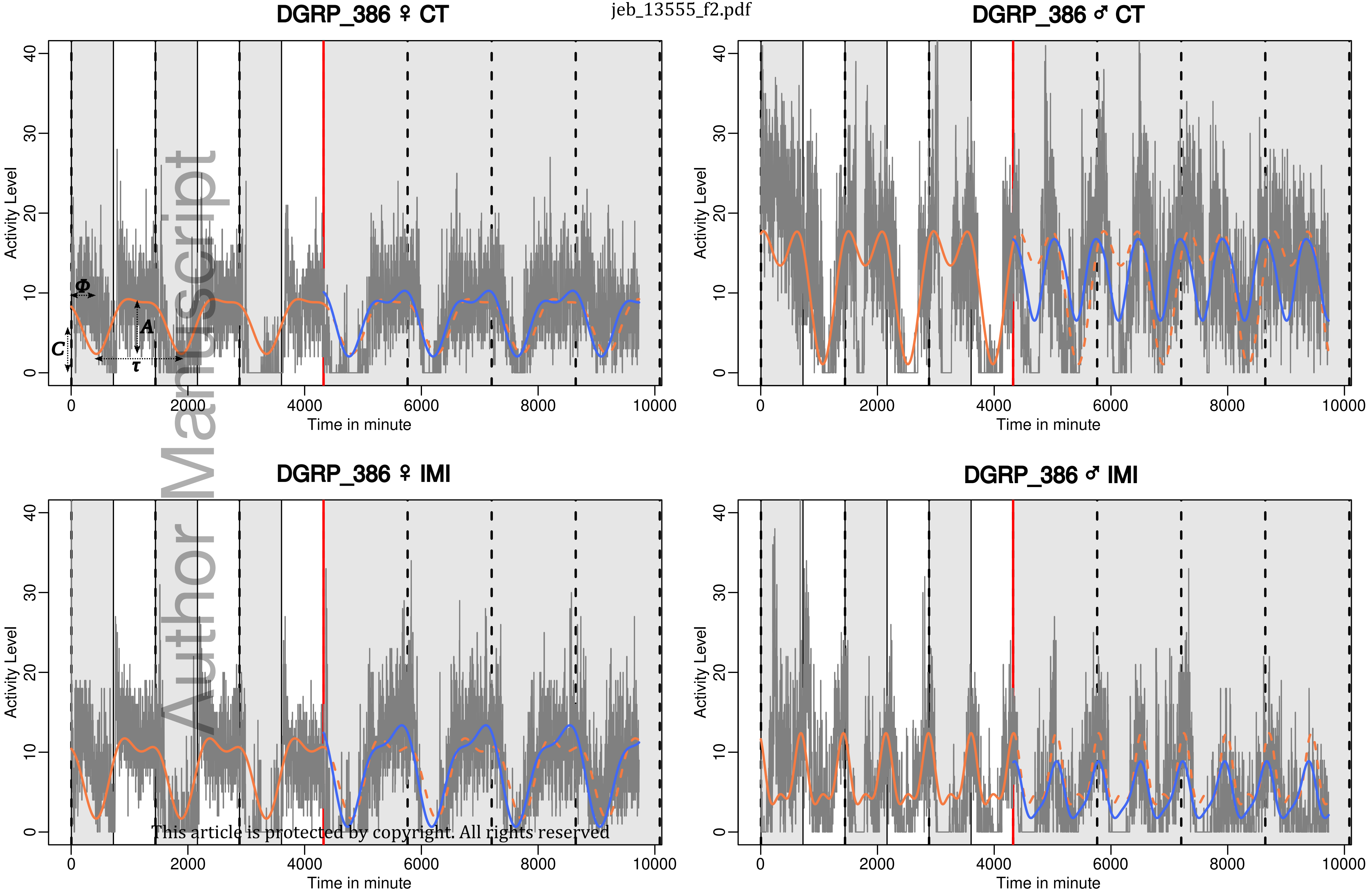
Figure 3: a) Factor map for the 16 activity traits analysed through PCA. b) PCA coordinates for individual observations. The crosses represent females, the closed circles represent males; red symbols represent imidacloprid-exposed individuals, turquoise symbols represent unexposed controls. **c) Genetic correlation for activity traits within and among insecticide treatment.** The genetic variances and covariances calculated using multi-trait mixed-linear models were normalised to correlations. The upper diagonal (trait names in red) represents values for imidacloprid-treated DGRP genotypes and the lower diagonal (trait names in turquoise) represents values for controls. Stars (*****) on the diagonal report significant difference (pairwise t-test, P<0.01) in correlations between a focal trait and the others traits depending on the exposure of the genotypes.

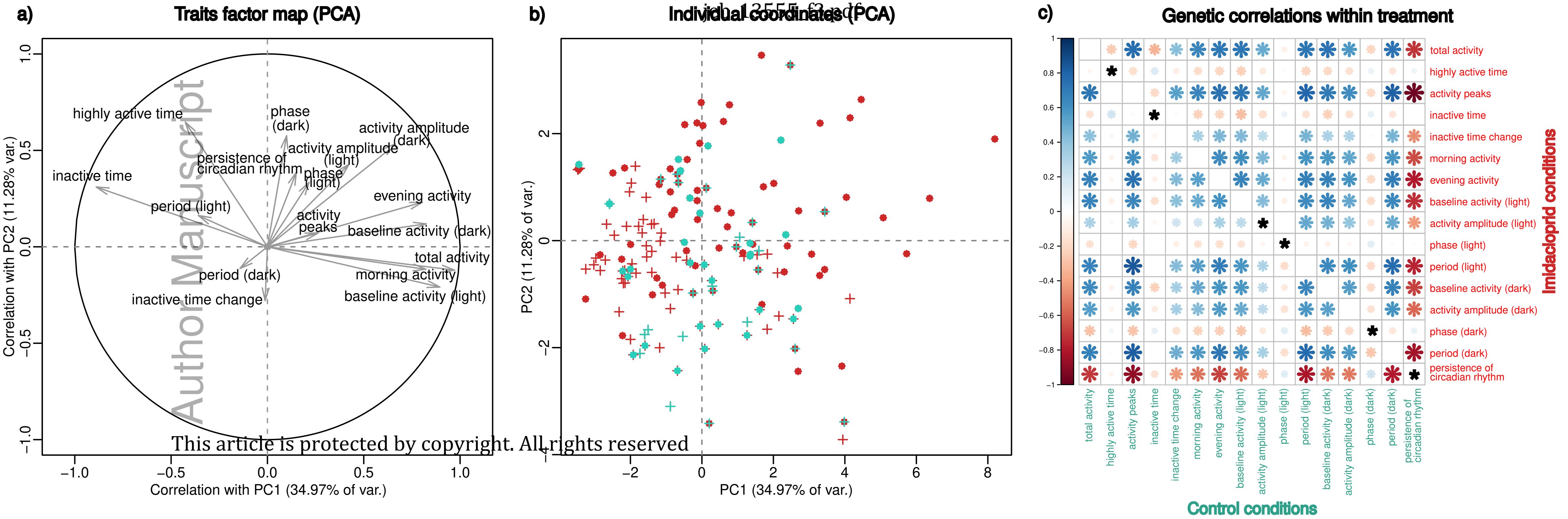
Figure 4: Progress in the courtship steps towards copulation over 10 minutes. The percentages along the y-axis report the proportion of the couples having completed each step. Each line represents the observation of a couple of flies going through a courtship assay, each dot represents the time of first occurrence (x-axis) of a courtship step (y-axis). Colours show the most advanced step completed by a couple, going from blue (orientation) to turquoise (singing) to green (licking) to attempted copulation (orange) until copulation success (red). Solid lines connect orderly succession of courtship steps, dashed lines denote connections that skipped a step. The top left-panel shows the correlation for control (CT) female and male, the bottom-left for imidacloprid-exposed (IMI) female and control male, the top-right for control female and imidacloprid-exposed male and the bottom-right for imidacloprid-exposed female and male.

Figure 5: Genetic correlation for courtship traits within and among insecticide treatment. The traits analysed were the likelihood of success (binary) modelled in a logistic framework. The genetic variances and covariances calculated using multi-trait mixed-linear models were normalised to correlations. The top left-panel shows the correlation for control (CT) female and male, the bottom-left for imidacloprid-exposed (IMI) female and control male, the top-right for control

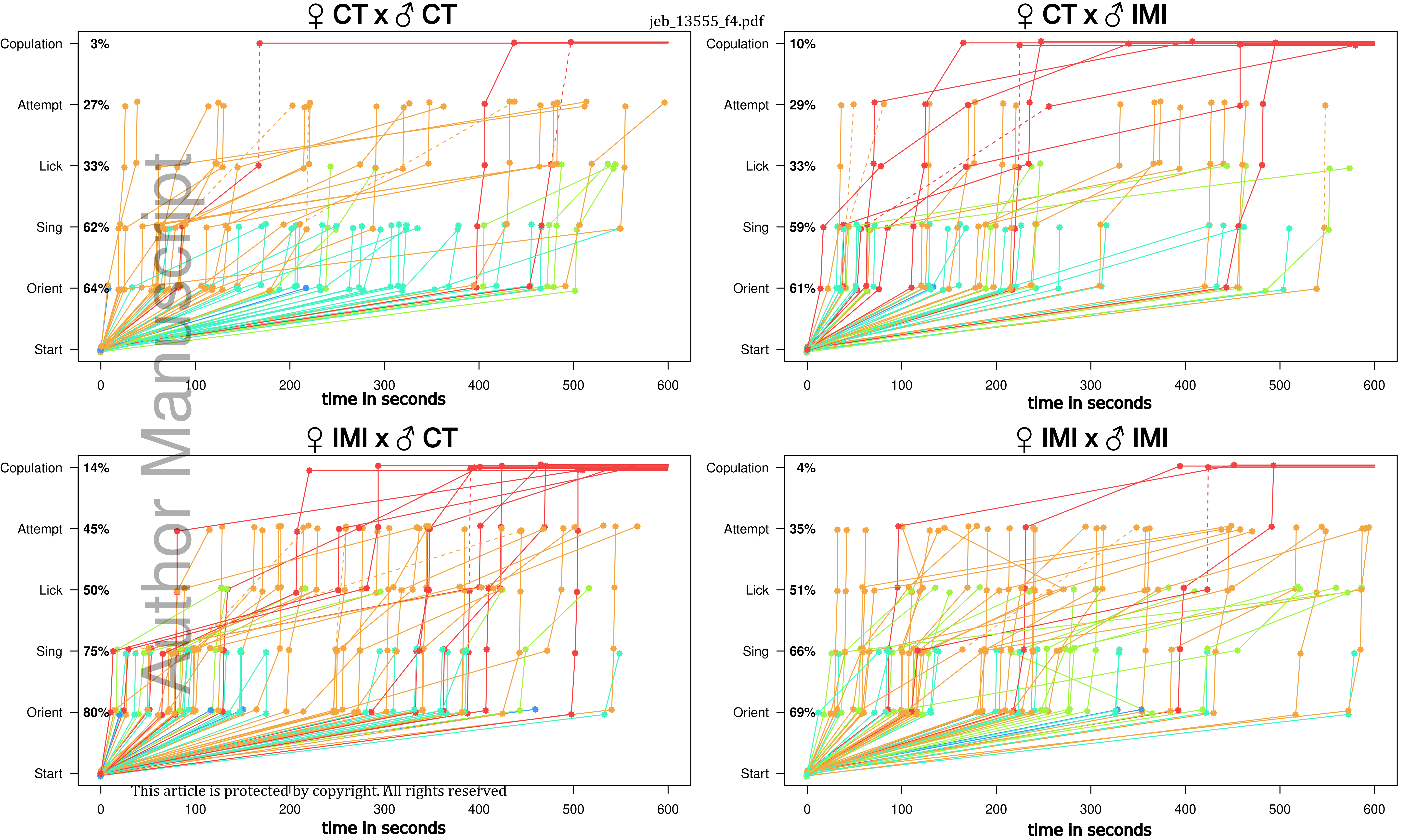
715 female and imidacloprid-exposed male and the bottom-right for imidacloprid-exposed female and male.

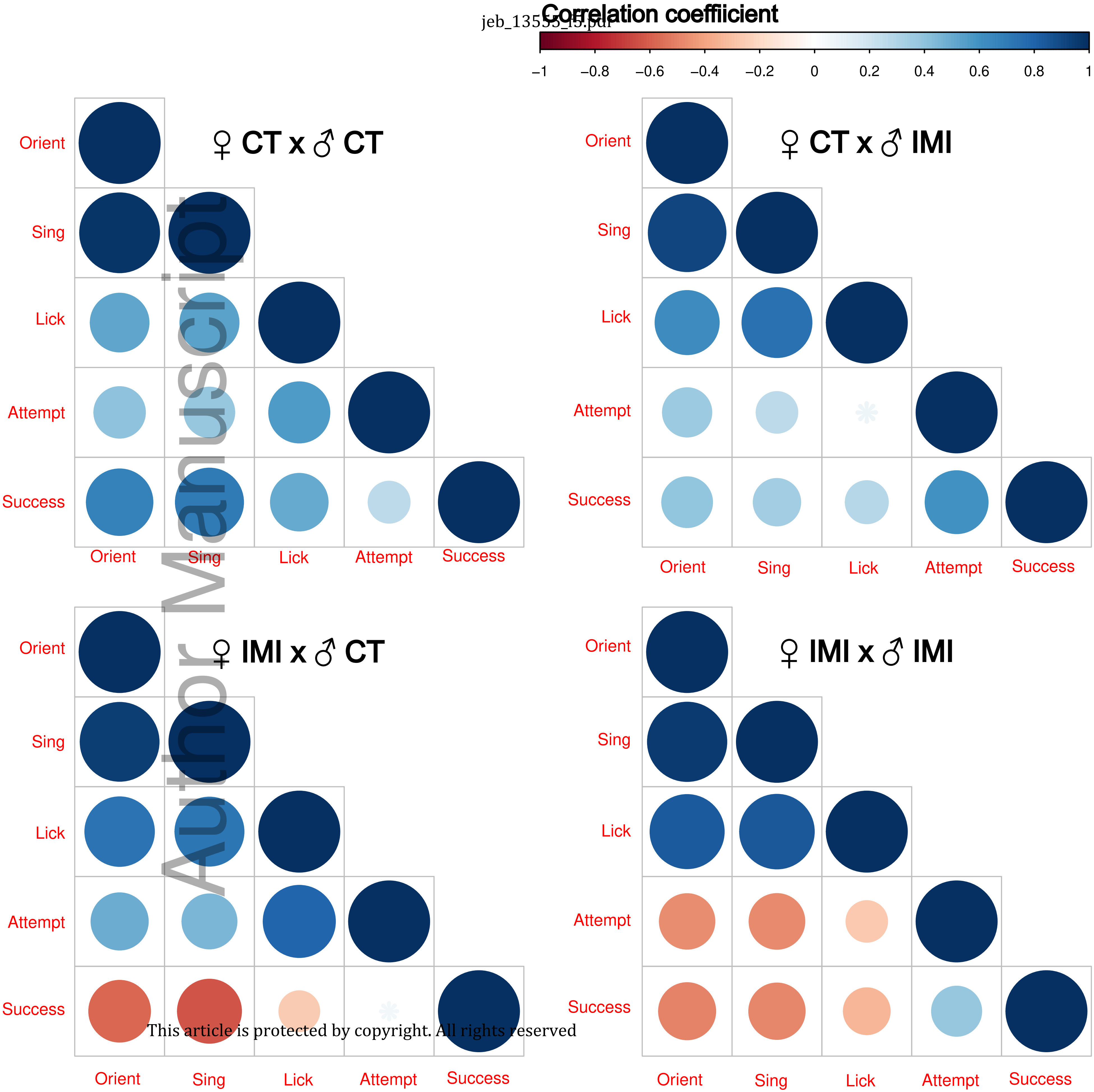

a
Determination of sublethal dose
Proportion of emerged flies
0
0.2
0.4
0.6
0.8
1
0
0.04
0.08
0.12
0.2
Imidacloprid concentration in ppm
b
Determination of resistance status (0.2ppm)
Proportion of emerged flies relative to population mean
0.4
0.2
0
-0.2
-0.4
Resistant
Susceptible
DGRP 217
DGRP 235
DGRP 280
DGRP 301
DGRP 359
DGRP 386
DGRP 439
DGRP 486
DGRP 509
DGRP 513
DGRP 589
DGRP 705
DGRP 776
DGRP 802
DGRP 843
DGRP 88
Genotype


DGRP_386 ♀ CT
DGRP_386 ♂ CT
DGRP_386 ♀ IMI
DGRP_386 ♂ IMI
Activity Level
0
10
20
30
40
Time in minute
0
2000
4000
6000
8000
10000
Φ
A
C
τ


a)
Traits factor map (PCA)
Correlation with PC1 (34.97% of var.)
Correlation with PC2 (11.28% var.)
−1.0
−0.5
0.0
0.5
1.0
highly active time
phase (dark)
activity amplitude (dark)
activity amplitude (light)
persistence of circadian rhythm
phase (light)
inactive time
evening activity
period (light)
activity peaks
baseline activity (dark)
total activity
period (dark)
morning activity
inactive time change
baseline activity (light)
b)
Individual coordinates (PCA)
PC1 (34.97% of var.)
PC2 (11.28% of var.)
−2
0
2
4
6
8
c)
Genetic correlations within treatment
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1
total activity
highly active time
activity peaks
inactive time
inactive time change
morning activity
evening activity
baseline activity (light)
activity amplitude (light)
phase (light)
period (light)
baseline activity (dark)
activity amplitude (dark)
phase (dark)
period (dark)
persistence of circadian rhythm
Imidacloprid conditions
Control conditions

jeb_13555_f4.pdf




Correlation coeffiicient
−1
−0.8
−0.6
−0.4
−0.2
0
0.2
0.4
0.6
0.8
1
♀ CT x ♂ CT
♀ CT x ♂ IMI
♀ IMI x ♂ CT
♀ IMI x ♂ IMI
Orient
Sing
Lick
Attempt
Success
*