The weakest link: Haploid honey bees are more susceptible to neonicotinoid insecticides

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Title:

The weakest link: Haploid honey bees are more susceptible to neonicotinoid insecticides

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Highlights:

- Neonicotinoid insecticides affected the developmental stability of honey bees
- Haploid males were more susceptible to neonicotinoids than diploid females
- Heterozygosity may play a key role in buffering insecticide exposure

Abstract

Neonicotinoid insecticides are currently of major concern for the health of wild and managed insects that provide key ecosystem services like pollination. Even though sublethal effects of neonicotinoids are well known, there is surprisingly little information on how they possibly impact developmental stability, and to what extent genetics are involved. This holds especially true for haploid individuals because they are hemizygous at detoxification loci and may be more susceptible. Here we take advantage of haplodiploidy in Western honey bees, *Apis mellifera*, to show for the first time that neonicotinoids affect developmental stability in diploid females (workers), and that haploid males (drones) are even more susceptible. Phenotypic fore wing venation abnormalities and fluctuating wing asymmetry, as measures of developmental instability, were significantly increased under field-realistic neonicotinoid-exposure of colonies. The higher susceptibility of haploid drones suggests that heterozygosity can play a key role in the ability to buffer the sublethal effects of neonicotinoids. Aiming to improve conservation efforts, our findings highlight the urgent need to better understand the role that genetics plays at enabling non-target organisms to cope with insecticide exposure.

Keywords:

Apis mellifera, xenobiotics, fluctuating asymmetry, wing venation, haplodiploidy, sublethal

1. Introduction

Insects are essential for the functionality of terrestrial ecosystems (Hallmann *et al.* 2017), and also play an important role in human food security such as by pollination of crops (Garibaldi *et al.* 2016). Therefore, the increasing global application of insecticides (Schreinemachers & Tipraqsa 2012) together with major declines of wild and losses of managed insect species (Neumann & Carreck 2010; Hallmann *et al.* 2017) has raised considerable concern over the stability of the ecosystem services provided by non-target insects (Potts *et al.* 2016). Neonicotinoids currently represent the most commonly employed insecticides in agricultural pest management practices (Simon-Delso *et al.* 2015). These insecticides act as antagonists of the nicotinic acetylcholine receptors, which leads to paralysis and ultimately death of the organism (Blacquière *et al.* 2012). Several studies have shown that they can induce sublethal effects on various invertebrates (Neumann *et al.* 2015), such as reducing fitness in solitary bees (Sandrock *et al.* 2014a), impairing bumble bee crop pollination services (Stanley *et al.* 2015), and comprising reproductive capacity of honey bee, queens and drones (Williams *et al.* 2015; Straub *et al.* 2016). Nevertheless, there is still an ongoing debate on the role of neonicotinoids for the decline of wild insect populations and losses of managed ones (Goulson 2013; Carreck & Ratnieks 2014), creating demand for an even better understanding of the sublethal effects of these agrochemicals. Even though the negative effects of other insecticides on morphogenetic development have been shown (Prado-Silva *et al.* 2018), there is surprisingly very little understanding of the potential effects of neonicotinoid insecticides on ontogenetic developmental stability.

Developmental stability is the ability of an organism to produce a consistent phenotype under any given environmental condition (Klingenberg 2019). Disruptions in development can have broad consequences (Willmore & Hallgrímsson 2005), including

reduced fitness (Møller 1997). Fluctuating asymmetry, described as random deviations from perfect asymmetry in bilateral traits, as well as the frequency of phenotypic abnormalities (e.g. wing venation abnormalities), are two common parameters used to measure developmental precision in insects (Lopuch & Tofilski 2016). Consequently, increased fluctuating asymmetry and phenotypic abnormalities suggest impaired developmental stability (Klingenberg 2019), and are regarded as surrogates for the health of insect populations (Beasley *et al.* 2013). Such developmental instability may be attributed to the disability of an individual to buffer against both genetic (e.g. mutations) or environmental factors (e.g. parasites, toxins) during morphogenesis (Müller *et al.* 2017; Gerard *et al.* 2018).

Past studies have shown that various stressors can cause fluctuating wing asymmetry, such as insecticides or pathogens (Chang *et al.* 2007; Gerard *et al.* 2018). Asymmetries in morphological structures imply perturbation in developmental homeostasis at the molecular, chromosomal and epigenetic levels, but underlying mechanisms are unknown (Klingenberg & Nijhout 1999). It is believed that genetic factors and physiological changes may play a crucial role in the ability to reach developmental stability (Chang *et al.* 2007), which includes managing xenobiotics by means of detoxifying enzymes (Derecka *et al.* 2013; Abbo *et al.* 2016). This may be particularly apparent in haploid individuals that are hemizygous at detoxification loci (O'Donnell & Beshers 2004) because heterozygosity likely enhances resistance towards parasitic and pathogenic stress (Baer & Schmid-Hempel 2003). Indeed, haploids were more susceptible when compared to their diploid counterparts under pathogen challenge (Retschnig *et al.* 2014; Strobl *et al.* 2019). Moreover, in various insect orders (Hymenoptera, Thysanoptera, and Coleoptera), males usually develop from unfertilized eggs and are therefore haploid (Evans *et al.* 2004), whereas females are diploid (Ross *et al.* 2019). In light of recent major declines of insects (Hallmann *et al.* 2017), the

possible higher susceptibility of haploid male insects may constitute a currently neglected gap in our knowledge despite the ample body of literature on insecticides. In particular, the possible role of heterozygosity for the developmental stability under neonicotinoid exposure is currently unknown.

For the first time, we compared susceptibility between the haploid male drone and diploid female worker honey bees to neonicotinoid insecticides by evaluating effects on developmental stability. The western honey bee (*Apis mellifera*) has historically served as a model organism to investigate the effects of environmental and anthropogenic stress on biological organisms, mainly because of their role as a keystone pollinator of agricultural and wild plants (Calderone 2012), as well as their well-known biology (EFSA 2014). We compared the frequency of phenotypic fore wing venation abnormalities and the degree of fluctuating fore wing asymmetry between diploid female worker and haploid male honey bees. Colonies were exposed to chronic field-realistic concentrations of two commonly applied neonicotinoid insecticides – thiamethoxam and its primary metabolite clothianidin (Blacquière *et al.* 2012). Based on previous studies indicating negative sublethal effects on worker larvae reared under neonicotinoid-exposure (López *et al.* 2017), we hypothesized that significantly increased levels of fore wing abnormalities and fluctuating wing asymmetry would be observed in both exposed workers and drones. Furthermore, we expected an increased degree of susceptibility in haploid drones when compared to diploid workers.

2. Material and Methods

2.1. Experimental set-up

The study was performed at the Hasli Ethological Station, Switzerland (46°58'03.359"N 7°23'54.703"E; WGS84 system) using 13 local queenright honey bee colonies established in April 2015 using the shook swarm method (Delaplane *et al.* 2013). Each colony was initially equipped with five frames containing organic Dadant-sized worker wax foundation (Supplementary data Figure S1), one laying sister queen, and ~1.8 kg of adult workers (Sandrock *et al.* 2014a; Williams *et al.* 2015).

2.2. Neonicotinoid exposure

In early May 2015, colonies were randomly allocated to one of two treatments – neonicotinoid (N=6) or control (N=7). According to established methods of in-hive insecticide exposure (Williams *et al.* 2015; Straub *et al.* 2016; Forfert *et al.* 2017), each colony was provided daily with 100 g pollen paste (60% fresh honey bee corbicular pollen, 10% organic honey, and 30% powder sugar) for 50 days to ensure that colonies were exposed for at least two complete brood cycles (Sandrock *et al.* 2014a; Straub *et al.* 2016). Pollen patties fed to colonies belonging to the neonicotinoid treatment additionally contained 4.9 ppb thiamethoxam and 2.1 ppb clothianidin (both Sigma-Aldrich), which represent field-realistic concentrations found in agricultural crops and neighboring wild plants (David *et al.* 2016; Tosi *et al.* 2018). Concentrations were confirmed by the French National Centre for Scientific Research using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Throughout the entire exposure period, each colony was fitted with a hive entrance pollen trap to promote pollen paste feeding by reducing forager-collected corbicular pollen (Human *et al.* 2013).

2.3. Source of drones and workers

To ensure that experimental drones were reared during treatment exposure, an additional frame containing organic drone cell wax foundation was added to each colony three weeks after initiation of pollen patty feeding (Rangel & Tarpy 2016). Eight days later, once drone-sized wax cells were drawn, each queen was caged to its colony's respective drone frame for 48 hours to obtain sufficient quantities of individuals of a known age cohort. Immediately after the 48 hour period, queens were placed on a worker brood frame within their colony using the previously described procedure to obtain equivalent worker cohorts. Each experimental drone and worker brood frame remained in its respective colony until it was transferred 24 hours prior to natural emergence to a laboratory incubator maintained at 34.5°C and 60% RH in complete darkness (Williams *et al.* 2013). To ensure that drones would successfully emerge and feed, 50 adult worker attendants from each colony were added to their respective drone frame (Currie 1987). Post-emergence, both experimental drones and workers remained on their respective brood frames for ~12 hours to ensure successful wing ventilation and maturation (Dickinson 1999). To ensure wing integrity, drones and workers were swiftly killed by carefully placing individuals into falcon tubes containing cotton soaked in ethyl acetate (Human *et al.* 2013). Falcon tubes were stored at -24°C until the wings of the individuals were examined.

2.4. Dissection and wing preparation

Dissection and preparation of experimental honey bee worker and drone wings were conducted during September 2017 and March 2018. Subsamples consisting of five individuals at a time were dissected and mounted to a cover slide to minimize possible negative effects caused by thawing. In total, 7-21 drones and 16-17 workers per colony were

assessed by dissecting each in a petri dish following Carreck *et al.* (2013). In brief, the thorax was carefully removed from the head and abdomen using dissection scissors. Then, the thorax was dissected longitudinally, to equally separating the left and right body side. Using a fine dissection scissor, the fore and hind wings were removed from the thorax to ensure that each wing base remained intact and could serve as a landmark for measurements (Klingenberg 2011). Fore wings were placed in EtOH of increasing concentrations (50, 70, 90%) for 5 min before rinsed in distilled water (Meixner *et al.* 2013), whereas the hind wings were not included for this study.

Fore wings were then carefully mounted on a prepared cover slide to prevent folds or air bubbles underneath the wings which would lead to distortions in landmark measurements (Klingenberg 2015). To facilitate digitalizing fore wings, each was positioned at the same height and orientation with the aid of a sliding caliper. Once mounted, 0.1 ml of the fixing agent Euparal (CarlRoth®) was carefully applied to each fore wing. Using a stereomicroscope (Olympus SZX7), possible undetected air bubbles in the solution and under the wings were gently removed using a forceps. Then, a coverslip [21 x 26 mm] was placed upon each individual fore wing before placing the slide in an incubator at 30°C for 24 h to dry.

2.5. Wing digitalization

Each individual fore wing (left and right) from 152 drones (94 controls and 58 neonicotinoids) and 212 workers (112 controls and 100 neonicotinoids) were analyzed at the Natural History Museum of Bern, Switzerland. Digital images were taken with a high-resolution camera mounted to a digital photo microscope (Keyence VHX 2000 digital photo microscope with VH-Z20R/W zoom lens). TPS photo files were built using the software

tpsUtil (Rohlf 2008).

2.6. Abnormalities in wing venation

Following Smith et al. (1997), all fore wing pairs were analyzed to identify wing venation abnormalities, also known as phenodeviants, such as vein protrusions, incomplete veins, as well as extra or missing veins (Smith *et al.* 1997; Lopuch & Tofilski 2016). These wing venation abnormalities are likely caused by genetic and/or environmental influences during the development of a particular trait (Smith *et al.* 1997; Lopuch & Tofilski 2016). In our study, we quantified the frequency of individual workers and drones from both treatment groups showing at least one wing venation abnormality. Furthermore, we quantified the total number of wing venation abnormalities present in both left and right wing for all individuals.

2.7. Morphometric measurements

The coordinates of 16 standardized, distinguishable landmarks located at intersections of the different veins and inter-vein areas were recorded and measured twice using the tpsDig software (Figure S2) (Rohlf 2001; Klingenberg 2015) to obtain a measurement error (ME) (Graham *et al.* 2010). Landmark coordinates were used to derive wing size and Procrustes coordinates that allowed the shape to be characterized, and the type of asymmetry to be determined (Klingenberg 2015). For drones, we obtained a vector containing 608 sizes corresponding to 152 individuals x 2 fore wings (right and left) x 2 replicates. For workers, we obtained a vector containing 848 sizes corresponding to 212 individuals x 2 fore wings (right and left) x 2 replicates. After normalizing all wing sizes, samples were aligned to directly compare their shape by obtaining a matrix of Procrustes coordinates containing the vector multiplied by 2 replicates (by 16 landmarks) and 2

dimensions (x and y).

In brief, the Procrustes approach extracted shape information from coordinate data in order to eliminate reflection (right and left wing) and variation in scale, position, and orientation among individual wings (Klingenberg 2015). First, reflection was removed by changing the sign of the x co-ordinates of all left wings and thereby flipping them horizontally to their mirror images. In a second step, all landmark configurations were scaled to the same centroid size. It is the centroid point (center of gravity) whose coordinates are the averages of the respective coordinates of all the landmarks (Klingenberg 2015). Thus, the position of each wing was standardized by superimposing centroids of all configurations on the origin (0, 0). The last step eliminated variation in orientation by rotating landmark configurations around their shared centroid to achieve an optimal fit of each wing to the consensus (average) configuration (Bookstein 1991).

2.8. Statistical Analyses

All statistical tests and figures were performed and produced using MorphoJ software (Klingenberg 2011) and NCSS v. 11.05 (NCSS 2018).

2.8.1 Wing venation abnormalities

Data were tested for normality by using the Shapiro-Wilk's test. We then applied a logistic regression model to determine significant differences among the frequencies of individuals with wing venation abnormalities from different treatments (control and neonicotinoid insecticide) and ploidy levels (diploid and haploid), wherein treatment and ploidy level were included as fixed terms and colony as a random effect. Total wing venation abnormalities per individual were compared using a Kruskal-Wallis ANOVA and post hoc

comparison (Bonferroni's test) to test for differences among treatments and sex.

2.8.2. Measurement error

Measurement error can cause a considerable degree of variation in asymmetry. Therefore, repeated measurements of the same individual fore wing were subject to a two-way mixed-model ANOVA [Side (fixed term) x Individual (random effect)] with repeated measurements of each side (Ondo *et al.* 2011). The fixed factor 'Side' tested for significant positive or negative differences between the left and right wing side of an individual regardless of its treatment group (i.e. directional asymmetry) (Graham *et al.* 2010). Significant effects of the random factor 'Individual' tested for differences in size or shape among individual drones and workers. Directional asymmetry in this context means a greater development of the wing on one side of the plane when compared to the other side (Klingenberg 2015). It can be quantified by calculating the size and shape difference between averages for left and right wings (Klingenberg 2015). The significance of the interaction term 'Individual x Side' tested for differences between sides among individuals (i.e. fluctuating asymmetry). Measurement error was considered negligible if the interaction term was significant ($P < 0.05$) and the mean square value for measurement error was substantially smaller than all other mean square values (Graham *et al.* 2010; Lopuch & Tofilski 2016).

2.8.3. Wing size and wing size asymmetry

Fore wing size was evaluated using centroid size [mm^2] (Dryden & Mardia 1989). This was calculated as the square root of the sum of the squared distances of the 16 landmarks from the centroid point (Klingenberg *et al.* 2001). Individual drone and worker fore wing size were defined as the average of the two replicates measured for left and right fore wings; this resulted in one wing size per side for each specimen. Wing size asymmetry was then

measured by calculating the difference between the sizes of the right and left fore wing (right-left (R-L)) (Palmer & Strobeck 2003). Mean value of wing size asymmetry obtained for each individual treatment group represented directional asymmetry, whereas, fluctuating asymmetry of wing size was calculated by subtracting directional asymmetry values from R-L values previously described (Klingenberg 2015). Colony (random effect) and treatment (fixed term) effects were examined using MANOVAs, whereas, differences in variation were established by using the F-test.

2.8.4. Wing shape and wing shape asymmetry

Analysis of fore wing shape was performed by aligning the configurations of landmarks using Procrustes superimposition (Bookstein 1991). Differences in wing shape between the treatment groups were examined using multivariate factorial ANOVA, with average Procrustes coordinates as random variables (individuals and colonies) and groups as fixed factors (treatments) (Nouvellet *et al.* 2011).

Because fluctuating asymmetry is the result of random irregularities during development, the magnitude of the shape asymmetry can be used as an estimate of developmental instability (Klingenberg 2015). Therefore, the Procrustes distance between left and right sides is a straightforward choice for measuring overall wing shape asymmetry. In the presence of directional asymmetry, which is a widespread phenomenon in animals (Pélabon & Hansen 2008), the Procrustes distance between the left and right sides is the combined result of fluctuating asymmetry and directional asymmetry. Because Procrustes distance is used to determine absolute shape differences, Mahalanobis distance, which compares levels of fluctuating asymmetry between groups, was also employed (Klingenberg 2015). Therefore, the Procrustes distance was used as a measure to determine absolute

shape differences, whereas the Mahalanobis distance measured differences between groups (i.e. sex and treatment groups) relative to the within-group variation (i.e. colony) (Klingenberg 2011; Lopuch & Tofilski 2016). Both Procrustes and Mahalanobis distances were calculated by using a canonical variate analysis (CVA), which maximizes the separation of specified groups and enables visualization of variation within treatments and sex (Klingenberg 2011). Procrustes and Mahalanobis distances, with respective significance (*P*) values for these distance, were generated using permutation tests (10,000 iterations per comparison); a scatter plot of the CVA scores was produced to visualize variation among treatments and sex (Benítez 2013).

3. Results

Summary statistics for all measured variables (i.e. the frequency of wing venation abnormalities, total wing venation abnormalities, centroid size [mean in mm^2], fluctuating asymmetry of wing size [log] and shape) in both worker and drone fore wings are provided in Table S1.

3.1 Wing venation abnormalities

3.1.1 Workers

No significant treatment effect was found between control (23.21 ± 5.05 %) and neonicotinoid-exposed (23.92 ± 4.30 %) workers for frequency of wing venation abnormalities (logistic regression, $R^2 = 0.008$, $df = 2$, $Z = -0.86$, $P = 0.39$) (mean ± standard deviation % (SD); Figure 1). Likewise, total wing venation abnormalities found in control (1 ± 1-2) and neonicotinoid-exposed (1 ± 1-2) workers did not significantly differ ($F_{1,49} < 0.01$, $P = 0.93$) (median ± 95% CI, Figure S3).

3.1.2 Drones

A significant difference was found between the frequency of wing venation abnormalities in control (44.53 ± 16.03 %) and neonicotinoid-exposed (70.35 ± 9.09 %) drones (logistic regression, $R^2 = 1.15$, $df = 3$, $Z = 4.85$, $P < 0.001$, Figure 1) (mean ± % SD). In contrast, no significant difference was observed for the total wing venation abnormalities found between control (2 ± 1-4) and neonicotinoid-exposed (2 ± 1-4) drones ($F_{1,79} < 0.2$, $P = 0.66$) (median ± 95% CI, Figure S3).

315 *3.1.3 Colony-level*

316 Irrespective of the treatment group, a significant difference of individuals showing
317 wing venation abnormalities (logistic regression, R^2 = 0.079, *df* =13, *Z* = -2.42, *P* < 0.015), as
318 well as total wing venation abnormalities ($F_{12,351}$ = 5.28, *P* = 0.009), was observed amongst
319 the 13 colonies.

320

321 *3.2 Measurement Error*

322 The two-way mixed-model ANOVA with repeated measures of each side in drones
323 and workers showed that mean square values were always substantially smaller than the
324 interaction term, and therefore highly significant (side x individual interaction; *P* < 0.001,
325 Table 1).

326

327 *3.3. Wing size and wing size asymmetry*

328

329 *3.3.1. Workers*

330 Neonicotinoid-exposure revealed no significant effect on worker wing size or wing
331 size asymmetry (F_1 = 3.07, *P* = 0.42, Figure 2A, C; Table 2). Wing size showed significant
332 individual (F_{198} = 28.11, *P* < 0.001; Table 2) and colony-level variation (F_{12} = 4.43, *P* < 0.001;
333 Table 2), with worker fore wings having a mean centroid size of 651.24 mm^2. Significant
334 directional asymmetry of wing size was observed (F_1 = 4.98, *P* < 0.03; Table 2 and 3), with the
335 right wings in both treatment groups on average ~0.014% larger than the left wings.
336 Regardless of treatment group, significant fluctuating asymmetry of wing size was observed
337 for all individuals (F_{211} = 57.14, *P* < 0.001; Tables 2 and 3).

338

339

340 *3.3.2. Drones*

341 Similar to workers, no significant treatment effect was observed for either wing size
342 or wing size asymmetry (F_1 = 0.48, P = 0.49, Figure 2B, D; Table 2). Significant individual
343 (F_{150} = 29.97, P < 0.001, Table 2) and colony-level variation (F_9 = 14.94, P < 0.001; Table 2)
344 were also observed for drone wing size; mean centroid size was 887.80mm^2. Significant
345 directional asymmetry of wing size was detected in drones (F_1 = 15, P < 0.002; Table 2 and 3),
346 with the right fore wing in both treatment groups ~0.067% larger than the left one.
347 Additionally, significant fluctuating asymmetry of wing size was observed in all experimental
348 drones (F_{152} = 69.94, P < 0.001; Table 2 and 3).

349

350 *3.4. Wing shape and wing shape asymmetry*

351

352 *3.4.1. Workers*

353 In contrast to wing size, a significant treatment effect on wing shape was observed
354 (F_{28} = 2.54, P < 0.001; Figure 2E, Table 2). Fore wing shape among all individuals (F_{554} = 13.13,
355 P < 0.001; Table 2) and colonies (F_{336} = 4.54, P < 0.001; Table 2) significantly differed.
356 Significant directional asymmetry of wing shape was observed for all individual workers
357 (F_{28} = 10.8, P < 0.001; Table 2). Regardless of treatment group, significant fluctuating
358 asymmetry of wing size was observed for all individuals (F_{5908} = 18.07, P < 0.001; Table 2). A
359 highly significant Procrustes distance of 0.0039 was revealed (P < 0.001; Table 4), with a
360 fluctuating asymmetry of wing shape between left and right wings being larger in
361 neonicotinoid-exposed workers (0.0028) than in controls (0.0030) (Procrustes distance;
362 Figure 3; Table 4). Likewise, a significant difference was also observed for the Mahalanobis
363 distance measured between treatment groups (P < 0.001; Table 4), which was 1.063. Control
364 and neonicotinoid-exposed workers showed significant fluctuating asymmetry of wing shape

between left and right wings of 0.666 and 0.721 respectively, which indicates increased shape asymmetry in the neonicotinoid-exposed workers (Mahalanobis distances, $P < 0.001$; Table 4).

3.4.2 Drones

Similar to workers, a significant effect of neonicotinoids on wing shape was observed ($F_{28} = 6.45$, $P < 0.001$, Figure 2F; Table 2). Again, fore wing shape among all individuals ($F_{4200} = 13.13$, $P < 0.001$; Table 2) and colonies ($F_{252} = 7.67$, $P < 0.001$; Table 2) significantly differed. Furthermore, a significant difference was observed for directional asymmetry of wing shape among individuals ($F_{28} = 1.95$, $P < 0.001$; Table 2). Significant fluctuating asymmetry of wing shape was also observed among experimental drones ($F_{4228} = 16.25$, $P < 0.001$; Table 2). A significant Procrustes distance of 0.0092 was revealed ($P < 0.001$; Table 4), with the fluctuating asymmetry of wing shape between left and right wings larger in neonicotinoid drones (0.0023) than in controls (0.0016) (Procrustes distance, Figure 3; Table 4). The Mahalanobis distance measured between treatment groups was 2.0247 and was significant ($P < 0.001$; Table 4). Control and neonicotinoid-exposed drones showed fluctuating asymmetry of wing shape between left and right fore wings of 0.409 and 0.599, respectively, which indicates increased shape asymmetry in neonicotinoid-exposed individuals (Mahalanobis distances; $P < 0.001$, Table 4).

3.5. Comparison between workers and drones

Drones had a significantly higher frequency of wing venation abnormalities compared to workers (logistic regression; $R^2 = 0.56$, $df = 2$, $Z = 2.2$, $P < 0.03$; Figure 1). In addition, drones experienced significantly more total abnormalities when compared to workers ($F_{3,128} = 6.68$, $P < 0.001$, Figure S3). A significant difference was observed between worker and

390 drone wing size (One way ANOVA; $P < 0.001$), with mean centroid wing size being 650.48 ±
391 11.48 mm^2 and 888.53 ± 17.15 mm^2 for the workers and drones, respectively (mean ± SD);
392 Table S1). Therefore, the average drone fore wing was 26.8% larger than that of a worker's.
393 Based on Procrustes distances, the CVA scores revealed significant differences in fore wing
394 shape between workers and drones as well as between treatments ($P < 0.001$), which
395 resulted in two non-overlapping clouds of points clearly discriminating workers from drones
396 (Figure 3). The sum of the first (97.04%) and second (2.45%) CV account for over (99.49%) of
397 the relative between-groups variation and accordingly, it was sufficient to examine only the
398 computed plot of the first two CVs. There was a significant difference for both the Procrustes
399 and Mahalanobis distance between workers and drones ($P < 0.001$; Table 4), with the
400 determined distances being 0.033 and 9.33, respectively. In addition, our results revealed
401 that the degree of fluctuating asymmetry of wing shape was significantly higher in drones
402 (0.0092) than in workers (0.0039) (Procrustes Distance, $P < 0.001$; Table 4).
403

4. Discussion

Our data show for the first time higher developmental instability of male haploid insects to neonicotinoids compared to diploid females. This provides empirical support to the haploid susceptibility hypothesis that points to increased susceptibility of haploids to environmental stressors (O'Donnell & Beshers 2004). Our data also provide general evidence that field-realistic concentrations of two widely employed neonicotinoid insecticides can negatively affect developmental stability in honey bees. Thiamethoxam and clothianidin significantly increased the frequency of wing venation anomalies, as well as the degree of fluctuating asymmetry in fore wings. This suggests that neonicotinoid insecticides may interfere with essential genetic cascades responsible for regulating developmental stability during larval insect development (De Celis & Diaz-Benjumea 2003), thereby contributing to the overwhelming evidence for effects of these chemicals on non-target organisms.

There is a consensus that abnormalities in wing venation patterns of insects are caused by both genetic and environmental factors (Mazeed 2011; Lopuch & Tofilski 2016). Regardless of the factor, the mechanisms explaining such wing venation abnormalities in holometabolous insects must occur during metamorphosis, when the rudiments of venation are still plastic (Truman & Riddiford 2019). Minor deviations or additional fragments can be interpreted as atavistic phenomena (Zanni & Opitz 2013), which were observed in our study for both honeybee drones and workers. Since control drones were more likely to show abnormalities compared to workers in the absence of any experimental environmental stressor, our results provide empirical support that recessive alleles can explain the higher frequency of such abnormalities (Bährmann 1963). This also suggests that minor wing abnormalities are unlikely to interfere with flight performance. Indeed, about 20% of workers had abnormalities in completely functional colonies, irrespective of neonicotinoid treatment.

The neonicotinoids revealed no significant effect on wing size in either workers or drones, whereas, they did have a significant negative effect on wing shape. Furthermore, our data showed that insecticide-exposure had no effect on wing size asymmetry, but did negatively affect wing shape asymmetry. Altered wing size asymmetry has been argued to significantly change insect flight efficiency as it causes a greater need for adjustment in beat frequency and orientation (Combes & Daniel 2003; Higginson & Barnard 2004). Similar consequences are likely to arise from increased wing shape asymmetry. Past studies investigating insecticide-exposure showed the contrary – increased fluctuating asymmetry for insect wing size, but not shape (Rosa *et al.* 2016; Gerard *et al.* 2018). Besides choice of insecticide studied (thiamethoxam and clothianidin versus imidacloprid (Rosa *et al.* 2016)), differences in insecticide-exposure route (in-hive pollen paste vs. water/nectar gathering in pesticide-treated fields (Ondo *et al.* 2011)), choice of model insect (honey bees versus damselflies or stingless bees (Chang *et al.* 2007; Rosa *et al.* 2016), and the varying life history and fundamental biology (Straub *et al.* 2015) may be reasons for the observed differences.

Interestingly, our data revealed that the fluctuating wing shape asymmetry was significantly higher in drones when compared to workers. Furthermore, the frequency of workers showing wing venation abnormalities, as well as the number of abnormalities present per individual, was not significantly influenced by neonicotinoid exposure. In sharp contrast, neonicotinoid exposure significantly increased the frequency, but not the total number of wing venation abnormalities in drones. Similar results for wing abnormalities and mean number of abnormalities have been reported for honey bees (Lopuch & Tofilski 2016). However, for the first time, we revealed an increased frequency of wing abnormalities and fluctuating wing asymmetry for haploid neonicotinoid-exposed drones when compared to diploid workers. This may be due to lack of heterozygosity at loci relevant during

metamorphosis. Indeed, the neonicotinoids may interfere with genetic pathways relevant for the development of wing veins (Bier 2000), because neonicotinoid-exposed bees also have to activate the detoxification pathways (Claudianos *et al.* 2006), such as cytochrome P450s or glutathione S-transferases (Li *et al.* 2007). The need for activation of additional gene cascades in exposed individuals may limit resources, which could otherwise be allocated to the core pathways essential for metamorphosis (Belles & Santos 2014; Truman & Riddiford 2019). As wing shape and wing venation patterns are governed by independent molecular mechanisms (Shimmi *et al.* 2014), our data suggest that neonicotinoid insecticides may negatively affect developmental homeostasis by interfering with at least two different mechanisms.

Even though neonicotinoid-exposed haploid drones were more susceptible, the observed degree of wing malformations in both controls and treatments is unlikely to interfere with flight abilities. Thus, the reported impaired flight ability of neonicotinoid-exposed bees (Blanken *et al.* 2015; Tison *et al.* 2016; Tosi *et al.* 2017) is more likely to result from an impact on behavior. This appears very plausible because neonicotinoids are neurotoxins in the first place (Blacquière *et al.* 2012).

Our data show that fore wing size and shape, as well as wing venation abnormalities, can significantly vary among colonies, regardless of treatment exposure. Considering that all colonies were maintained under the same conditions (common garden approach), it appears likely that genetic variation may be responsible. Such variation in sensitivity towards insecticides is known (Miyo *et al.* 2000). Yet, our results provide further evidence that the detoxification capacities in honey bee colonies can significantly vary (Sandrock *et al.* 2014b) and subsequently highlight the importance of considering both individual and colony-level genetics when interpreting data of toxicological studies. Moreover, the data confirm a

476 significant level of directional asymmetry of wing size and shape, in favor of larger right
477 wings. Differences in left and right body sides have already been observed in several haploid-
478 diploid insects (Klingenberg 2019), and is known to be widespread throughout the animal
479 kingdom (Palmer 1996). These differences are most likely explained by the existence of a left-
480 right axis that reflects distinct positional identities of the wing imaginal discs on either body
481 side (Klingenberg *et al.* 1998); however, the overall small magnitude of directional
482 asymmetry precludes directional asymmetry from having any major adaptive role or
483 consequences (Pélabon & Hansen 2008).

484 In conclusion, our data show that neonicotinoids can interfere with honey bee
485 metamorphosis and that haploids are more susceptible. This creates demand for a better
486 understanding of the possible impact of these insecticides on other factors during
487 metamorphosis (e.g. spermatogenesis (Straub *et al.* 2016)). Further, haplodiploid insect
488 species and genetically less diverse populations may be more at risk, which seems relevant
489 for conservation efforts (Reed & Frankham 2003; Winfree 2010). Future risk assessments
490 should, therefore, consider the genetic basis of susceptibility to insecticides and the weakest
491 link within a species.

492

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Data accessibility: The complete raw data can be found on the Dryad repository (DOI link provided upon acceptance).

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Authors' Contributions: L.S., G.R.W. & P.N. conceived the study; A.F., S.B., G.R.W. & L.S. carried out the experiment and collected data; P.N. provided materials; A.F. & L.S. designed the statistical analysis and wrote the statistical methods and analyzed data; All authors contributed to the interpretation of results and writing of the manuscript.

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List of captions:

Figure 1: Effects of neonicotinoid insecticides on honey bee, *Apis mellifera* (L.), worker and drone fore wing venation. For each of the four treatment groups, the frequency of wing venation abnormalities was measured. The bar charts show the overall observed frequency of individuals with at least one wing venation abnormality in either left or right wing as well as the mean ± standard error. A significant difference (logistic regression, $P < 0.05$) between treatments and sex is indicated by different letters (A, B, C).

Figure 2: Effects of neonicotinoid insecticides on honey bee, *Apis mellifera*, worker (A, C, E) and drone (B, D, F) fore wing size and shape. All variables were measured using 16 landmarks from each fore wing. Wing size was evaluated using the centroid size [mm^2, A & B) whereas fluctuating asymmetry of wing size (centroid size [log], C & D) was then measured by calculating the difference between the sizes of the right and left fore wings (R-L). No significant treatment effect was observed either workers or drones for either of these parameters ($P > 0.05$). Analysis of fore wing shape was performed by aligning the configurations of landmarks using Procrustes superimposition (Procrustes distance (P.D.) in E & F). Significant differences were observed for both workers and drones ($P < 0.001$). The boxplots show the inter-quartile-range (box), the median (black line within box), and outliers (dots). A significant difference ($P < 0.001$) between treatments is indicated by ***.

Figure 3: Scatter plot of the first two canonical variates for honey bee (*Apis mellifera* (L.)) drones and workers exposed to neonicotinoid insecticides. Canonical variate analysis (CVA) scores with 95% confidence ellipses of the Procrustes analysis of treatment (control vs. neonicotinoid) and sex (worker vs. drone). Based on the Mahalanobis distances, a significant

733 difference was observed between treatments groups for both workers and drones
734 ($P < 0.001$), and a significant difference was observed between all workers and drones
735 regardless of treatment ($P < 0.001$). Large circles indicate clusters of treatments and
736 individual workers and drones. A significant difference ($P < 0.001$) of wing shape is indicated
737 by ***.

738

739 **Table 1: Calculated measurement errors using Procrustes ANOVA of centroid size and shape**
740 **of honey bee, *Apis mellifera* (L.), drone and worker fore wings.** Results obtained from the
741 Procrustes analysis were used to assess measurement error on the fore wing samples that
742 had been measured twice. The analyses evaluated five effects for both centroid size and
743 shape: 1. 'Sex' - female workers or male drones, 2. 'Individual' - tested for differences among
744 individuals (N = 364), 3. 'Side' - tested for directional asymmetry within individuals (i.e. one
745 wing side consistently different from the other), 4. 'Individual x Side' - tested for differences
746 in fluctuating asymmetry among individuals, and 5. Measurement error was not of concern
747 as the 'Individual x Side' interaction was highly significant and the corresponding mean
748 square substantially exceeded measurement error.

749

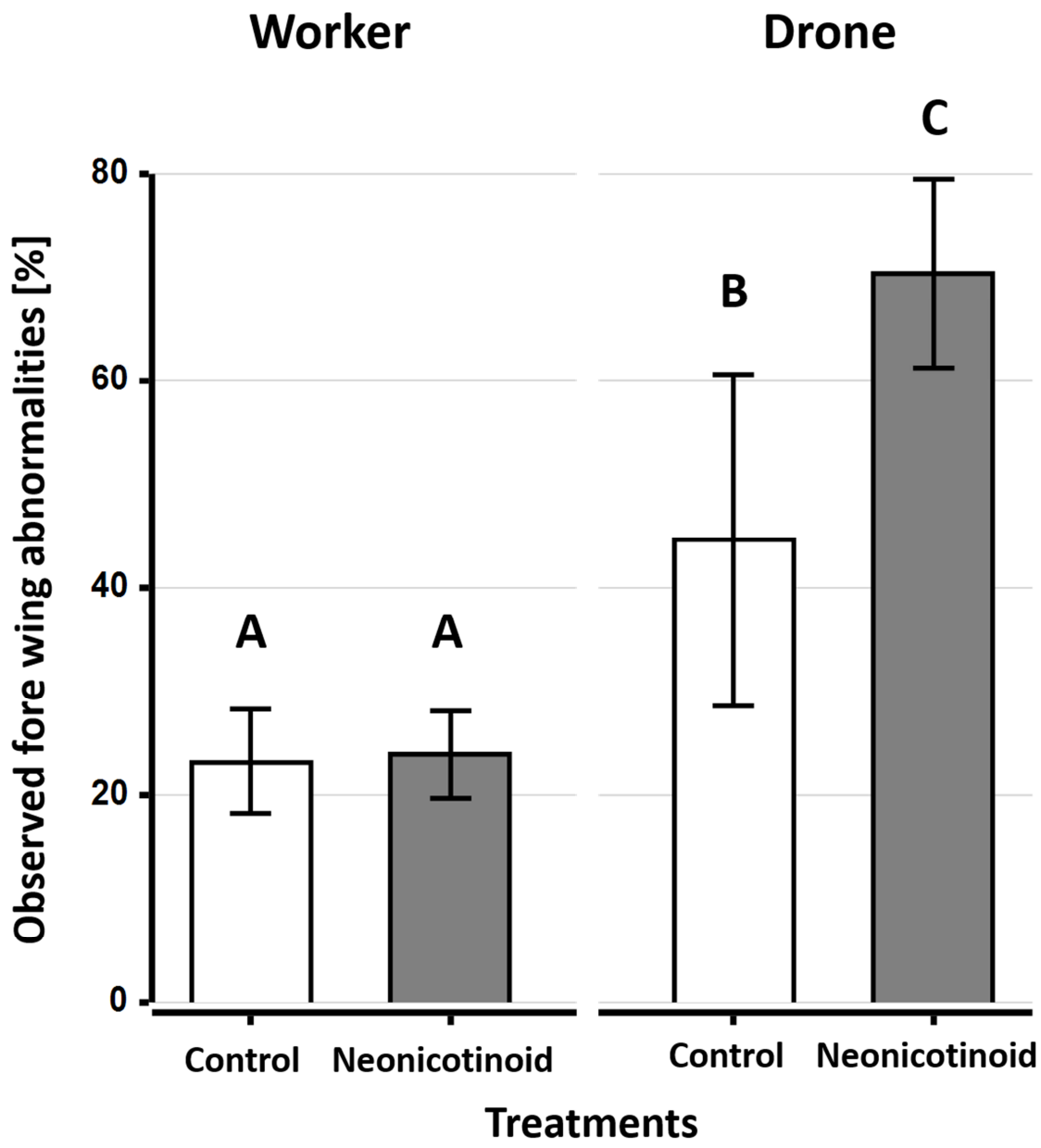
750 **Table 2: Summary of honey bee drone and worker, *Apis mellifera* (L.), fore wing size and**
751 **shape for control and neonicotinoid treatment groups.** Degrees of freedom (df), the sum of
752 squares, mean squares, and F and *P*-values are presented for random effects 'Individual' and
753 'Colony', as well as for fixed effects 'Treatment' (control and neonicotinoid) and 'Side' (e.g.
754 right and left fore wing). The interaction term was included to indicate the level of fluctuating
755 asymmetry. Treatment effects were observed for shape ($P < 0.001$) but not size ($P = 0.4190$
756 for workers, $P = 0.4906$ for drones).

Table 3: Summary of obtained sizes for honey bee, *Apis mellifera* (L.), drone and worker fore wings, and mean asymmetry of fore wing size. Centroid size represented fore wing size and was calculated by the square root of the sum of the squared distances of the 16 landmarks from the centroid (center of gravity). Results revealed that the right wing was larger than the left wing for both treatment groups, which resulted in a significant degree of fluctuating asymmetry of wing size for both controls and neonicotinoids ($P < 0.001$).

Table 4: Summary and comparison of Procrustes distances measured for honey bee, *Apis mellifera* (L.), worker and drone fore wings in control and neonicotinoid treatment groups. The degree of fluctuating shape asymmetry between left and right fore wing of each treatment was measured using Procrustes distances. Neonicotinoid-exposed workers and drones revealed a significantly higher degree of Procrustes distances ($P < 0.001$).

771

772 **Figures and Tables:**



773

774 Figure 1:

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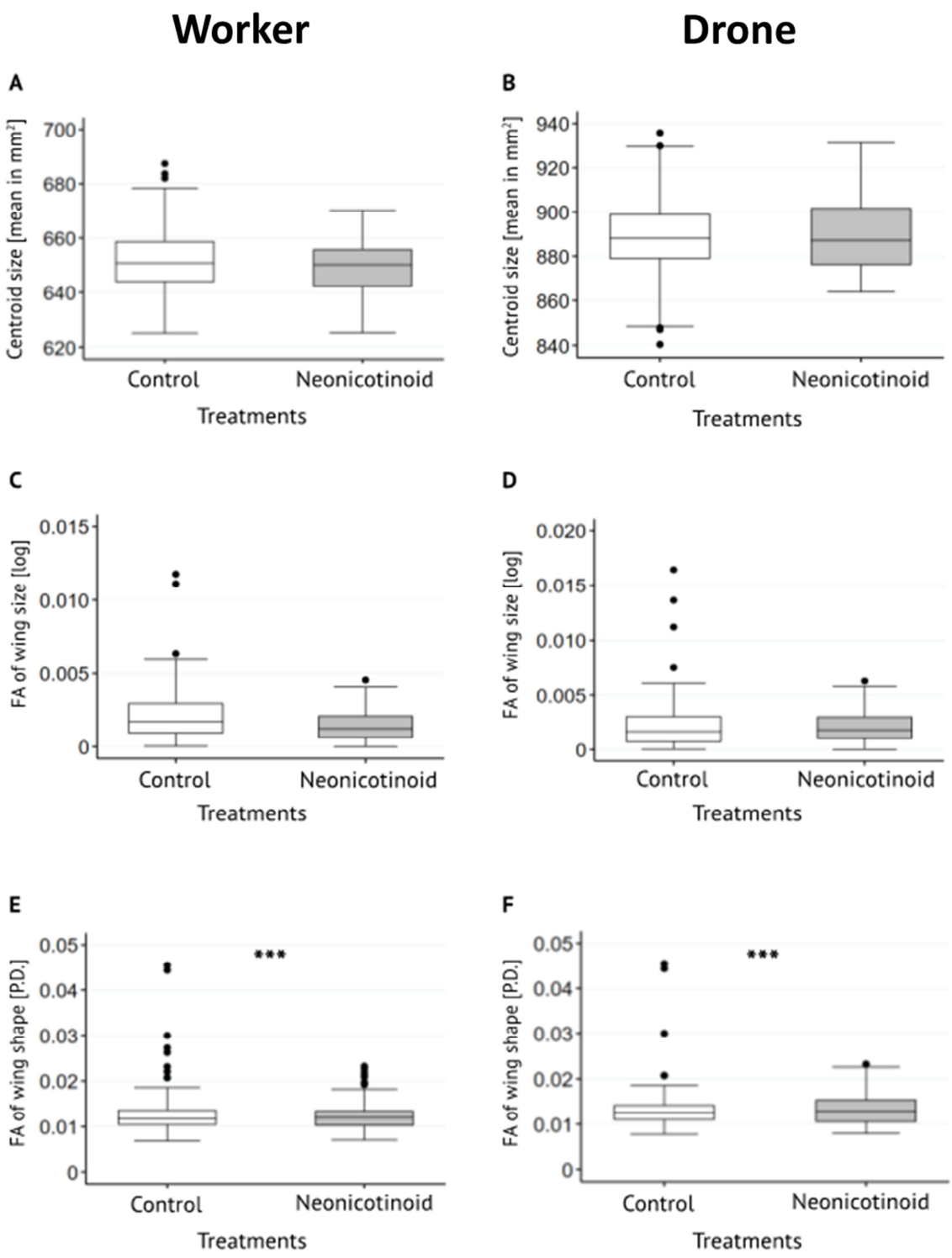
Worker
Drone
A
B
C
D
E
F
Centroid size [mean in mm²]
FA of wing size [log]
FA of wing shape [P.D.]
Control
Neonicotinoid
Treatments
***

Figure 2

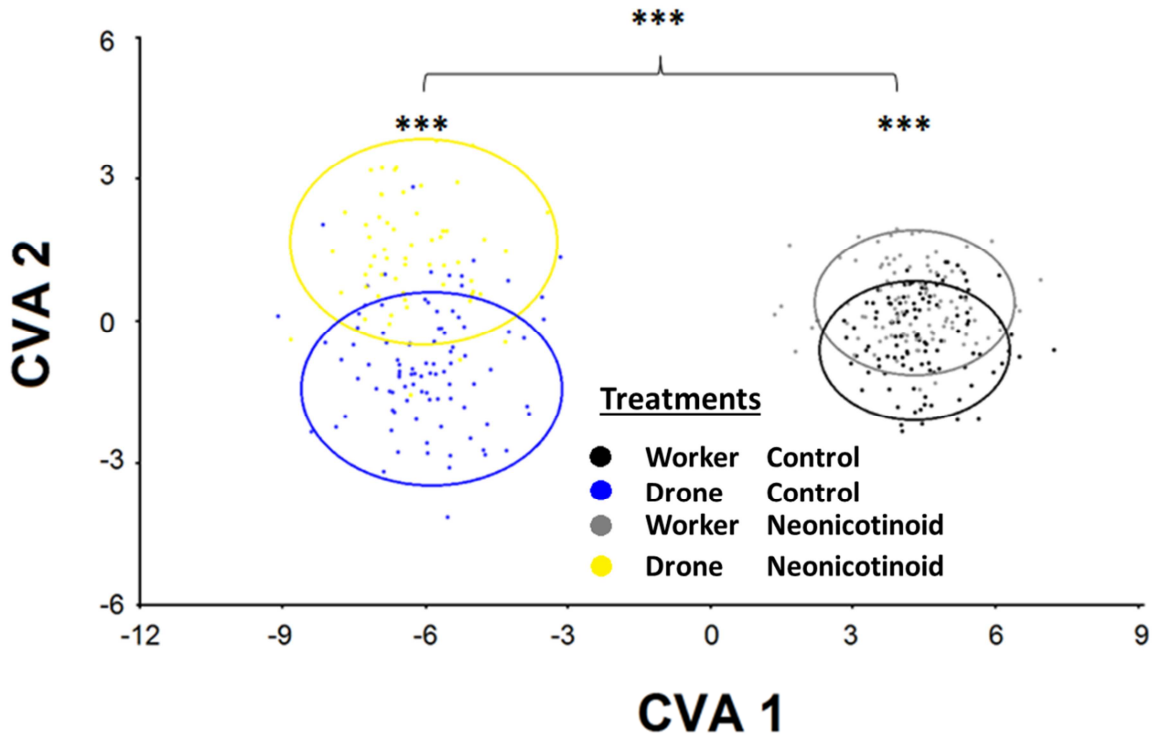

Figure 3

Table 1

	Fore wing			
	Mean Square (MS)	**df**	**F**	***P* (param.)**
Centroid size [MS in mm²]				
Sex	2670218.88	1	2448.64	< 0.0001
Individual	1090.49	362	31.7	< 0.0001
Side	602.30	1	17.51	< 0.0001
Individual x Side	34.40	363	62.92	< 0.0001
Measurement Error	0.55	727		
Shape [Procrustes MS]				
Sex	0.0139904	28	193.15	< 0.0001
Individual	0.0000724	10136	11.88	< 0.0001
Side	0.0000519	28	8.51	< 0.0001
Individual x Side	0.0000061	10164	17.19	< 0.0001
Measurement Error	0.0000004	20356		

:

Table 2:

Centroid Size

Sex	Source	Sum of Square (SS)	Mean Square (MS)	df	F	*P*
Worker						
	Treatment	2640.75	264.75	1	3.07	0.4190
	Colony	45772.44	3814.37	12	4.43	< 0.0001
	Individual	170406.12	86.64	198	28.11	< 0.0001
	Side	152.48	152.48	1	4.98	0.0267
	Individual x Side	6460.86	30.62	211	57.14	< 0.0001
Drone						
	Treatment	305.38	305.38	1	0.48	0.4906
	Colony	85947.93	9549.77	9	14.94	< 0.0001
	Individual	176079.34	1173.86	150	29.97	< 0.0001
	Side	587.68	587.68	1	15	0.0020
	Individual x Side	5914.91	39.17	151	69.94	< 0.0001

Shape

Worker						
	Treatment	0.003231	0.000115	28	2.54	< 0.0001
	Colony	0.069355	0.000206	336	4.54	< 0.0001
	Individual	0.252322	0.000046	554	8.67	< 0.0001
	Side	0.001588	0.000057	28	10.8	< 0.0001
	Individual x Side	0.031028	0.000005	5908	18.07	< 0.0001
Drone						
	Treatment	0.012217	0.000436	28	6.45	< 0.0001
	Colony	0.130821	0.000519	252	7.67	< 0.0001
	Individual	0.000095	0.000095	4200	13.13	< 0.0001
	Side	0.000393	0.000014	28	1.95	0.0024
	Individual x Side	0.000007	0.000007	4228	16.25	< 0.0001

Table 3:

			Centroid Size (log)			Wing Size Asymmetry (log)		
			Mean	Standard Error	*P*	Mean	Standard Error	*P*
Sex	**Treatment**	**Wing Side**						
Worker	**Control**	**Right**	2.8139	1.0588	0.0316	0.0022	0.0019	<0.001
		Left	2.8136	1.0630				
	Neonicotinoid	**Right**	2.8125	0.9966	0.0081	0.0014	0.0010	<0.001
		Left	2.8120	0.9760				
Drone	**Control**	**Right**	2.9488	1.2455	0.0107	0.0024	0.0027	<0.001
		Left	2.9478	1.2677				
	Neonicotinoid	**Right**	2.9495	1.2125	0.0016	0.0021	0.0015	<0.001
		Left	2.9485	1.2113				

Table 4:

		Procrustes Distances			*P* -values for Procrustes Distances			Mahalanobis Distances			*P* -values for Mahalanobis Distances		
	Sex		0.033			< 0.001			9.3268			< 0.001	
	Treatment		0.0039			< 0.001			1.063			< 0.001	
	Treatment and Side	**Control Left**	**Control Right**	**Neonicotinoid Left**	**Control Left**	**Control Right**	**Neonicotinoid Left**	**Control Left**	**Control Right**	**Neonicotinoid Left**	**Control Left**	**Control Right**	**Neonicotinoid Left**
Worker	**Control Right**	0.0028			0.02			0.666			< 0.0001		
	Neonicotinoid Left	0.0041	0.0044		< 0.0001	< 0.0001		1.1055	1.1855		< 0.0001	< 0.0001	
	Neonicotinoid Right	0.0051	0.004	0.003	< 0.0001	< 0.0001	< 0.0001	1.3089	1.0645	0.7211	< 0.0001	< 0.0001	< 0.0001
	Treatment		0.0092			< 0.001			2.0247			< 0.001	
	Treatment and Side	**Control Left**	**Control Right**	**Neonicotinoid Left**	**Control Left**	**Control Right**	**Neonicotinoid Left**	**Control Left**	**Control Right**	**Neonicotinoid Left**	**Control Left**	**Control Right**	**Neonicotinoid Left**
Drone	**Control Right**	0.0016			0.99			0.4089			0.99		
	Neonicotinoid Left	0.0092	0.0099		< 0.0001	< 0.0001		2.2695	2.3827		< 0.0001	< 0.0001	
	Neonicotinoid Right	0.0088	0.0093	0.0023	< 0.0001	< 0.0001	0.97	2.1859	2.2818	0.5991	0.0004	0.0001	0.9981

Title:

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