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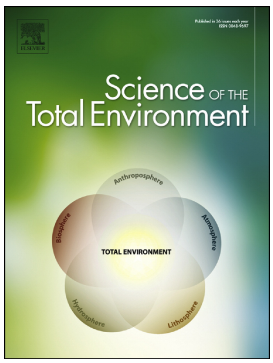

Buffered fitness components: Antagonism between malnutrition and an insecticide in bumble bees

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Title: Buffered fitness components: antagonism between malnutrition and an insecticide in bumble bees

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Abstract:

Global insect biodiversity declines due to reduced fitness are linked to interactions between environmental stressors. In social insects, inclusive fitness depends on successful mating of reproductives, i.e. males and queens, and efficient collaborative brood care by workers. Therefore, interactive effects between malnutrition and environmental pollution on sperm and feeding glands (hypopharyngeal glands (HPGs)) would provide mechanisms for population declines, unless buffered against due to their fitness relevance. However, while negative effects for bumble bee colony fitness are known, the effects of malnutrition and insecticide exposure singly and in combination on individuals are poorly understood. Here we show, in a fully-crossed laboratory experiment, that malnutrition and insecticide exposure result in neutral or antagonistic interactions for spermatozoa and HPGs of bumble bees, *Bombus terrestris*, suggesting strong selection to buffer key colony fitness components. No significant effects were observed for mortality and consumption, but significant negative effects were revealed for spermatozoa traits and HPGs. The combined effects on these parameters were not higher than the individual stressor effects, which indicates an antagonistic interaction between both. Despite the clear potential for additive effects, due to the individual stressors impairing muscle quality and neurological control, simultaneous malnutrition and insecticide exposure surprisingly did not reveal an increased impact compared to individual stressors, probably due to key fitness traits being resilient. Our data support that stressor interactions require empirical tests on a case-by-case basis and need to be regarded in context to understand underlying mechanisms and so adequately mitigate the ongoing decline of the entomofauna.

Keywords: gland, interaction, malnutrition, neonicotinoid thiamethoxam, pollen, sperm

1. Introduction

Pollinators play a crucial role for terrestrial ecosystems and food security as they are indispensable in sustaining wild plants and crops (Klein et al., 2007). With ~20'400 known species globally (Engel et al., 2020), bees are among the most important pollinators (Potts et al., 2016, 2010). However, there is clear evidence for widespread declines for various wild bee species and losses of managed bees (Neumann and Carreck, 2010; Carvalheiro et al., 2013; Nieto et al., 2014; Goulson et al., 2015). These reports have sparked much concern as the consequences of pollinator declines will almost certainly result in negative downstream effects for both environmental and human health (Potts et al., 2016). A wide array of environmental factors likely govern bee declines and losses, including habitat loss (Marshall et al., 2017), pest and pathogens (Neumann and Carreck, 2010; Ravoet et al., 2014), climate change (Soroye et al., 2020), and intensified industrial agriculture (Winfree, 2010). Arguably, two pivotal factors that impair wild bee fitness and subsequently drive population declines are the ubiquitous exposure to xenobiotic chemicals and the increasing lack of food (Baude et al., 2016; Straub et al., 2020b, Topping et al., 2021).

Global agricultural intensification has resulted in the loss of natural habitats and the simplification of landscapes (Tilman et al., 2002; Foley et al., 2005), leading to fewer natural nesting sites as well as the reduction in floral resources and diversity (Kremen et al., 2002). Furthermore, the application of agrochemicals used to enhance crop yields poses an inadvertent threat to non-target organisms (Neumann, 2015; Hayes and Hansen, 2017). Indeed, the ubiquitous use of systemic neonicotinoids, one of the most widely applied classes of insecticides globally (Bass et al., 2015), has become a serious concern for pollinators as residues are frequently found contaminating pollen and nectar of flowering

plants and crops (Bonmatin et al., 2015; David et al., 2016). Due to their non-specific mode of action (Matsuda et al., 2020), lethal as well as a range of adverse sublethal effects including altered consumption behaviour (Cresswell et al., 2012), reduced glandular development (Hatjina et al., 2013), and impaired reproductive physiology (Williams et al., 2015; Straub et al., 2021b; Strobl et al., 2021) are well documented in bees. Subsequently, in 2013 the EU Commission implemented severe restrictions for the outdoor use of plant protection products and treated seeds containing three neonicotinoid substances to safeguard non-target insects (clothianidin, imidacloprid, and thiamethoxam) (EU-Commission, 2013). In addition, the application of these neonicotinoids was prohibited in crops consider to be particular bee-attractive, such as maize, oilseed rap or sunflower. However, the moratorium did not ban the use of these pesticides in crops such as sugar beets or winter-sown cereals, from which residues have been shown to spread to nearby wild plants (David et al., 2016). Besides neonicotinoid exposure, a lack of pollen, which is the primary source of dietary protein and lipids (de Groot, 1952), can adversely affect individual bee longevity (Smeets and Duchateau, 2003), hypopharyngeal gland development (Pernal and Currie, 2000), as well as impair key physiological process such as immunocompetence and detoxification abilities (Alaux et al., 2010; Di Pasquale et al., 2013). In addition, pollen deprivation can affect female reproduction by reducing ovary activation as well as fecundity (Moerman et al., 2017; Stuligross and Williams, 2020). Similar data on how nutritional quality and quantity may influence male insect reproductive physiology remains vastly unexplored (Leather, 2018). Thus, unraveling the potential effects of inadequate nutrition and xenobiotic exposure in agricultural systems is critical for pollinator conservation efforts because the likelihood of encountering both stressors simultaneously is almost certain. Yet,

our current understanding of how these two common stressors interact to affect bee health remains scarce (Siviter et al., 2021).

The nature of an interaction can vary depending on the choice of environmental stressors, model species, and the assessed endpoint measurement (Straub et al., 2020a; Bird et al., 2021; Siviter et al., 2021), and may range from being additive (i.e., the effect of two individual stressors equal their combined effects), to synergistic (i.e., the combined effect is greater than the predicted additive effect), to antagonistic (i.e., combined effect of two stressors is smaller than the predicted additive effect) (Folt et al., 1999; Côté et al., 2016). As proteins and amino acids found in pollen are crucial to modulate detoxification abilities in bees (Di Pasquale et al., 2013), inadequate pollen recourse can increase the susceptibility towards pesticide exposure (Barascou et al., 2021). Therefore, negative effects are assumed in bees simultaneously exposed to nutritional stress (i.e., pollen deprivation) and pesticides. Indeed, studies evaluating the potential effects of these two stressors have revealed both synergistic and additive negative effects on lethal (Linguadoca et al., 2021; Tosi et al., 2017) and sublethal endpoint measurements (Schmehl et al., 2014; Stuligross and Williams, 2020), respectively. Given that similar negative effects are observed on key reproductive physiological parameters in bumble bees, such as on the glands required for digestion and brood care (i.e., hypopharyngeal glands (HPGs)) or sperm traits (i.e., spermatozoa counts and viability), this may provide an additional plausible mechanistic explanation for recent population declines (Bommarco et al., 2012; Colla et al., 2012). However, the impact of any given stressor can vary depending upon the level, e.g. in ants additive effects of virus and pestidice were observed at the level of individuals and castes, while co-exposure with both stressors elicited antagonistic effects on colony size (Schläppi et al., 2021). Further, some

traits may be more buffered than others depending their relevance to fitness, where traits closely linked to fitness are expected to be more resilient to stochastic environmental change (Morris and Doak, 2004).

Here, we measured the individual and combined effects of pollen deprivation (hereafter malnutrition) and a high, yet field-realistic, neonicotinoid exposure on survival, consumption, and fitness-relevant physiological traits (i.e., HPG acini width and spermatozoa counts and viability) in bumble bees, *Bombus terrestris*. We accomplished this by using a previously established method (Minnameyer et al., 2021), where adult *B. terrestris* workers and drones were maintained individually under laboratory conditions. We used the neonicotinoid insecticide thiamethoxam, which despite its restricted usage in Europe for outdoor purposes, remains frequently applied globally (Thompson et al., 2020), leading to contaminations occasionally exceeding concentrations of 20 ng g^{-1} (Botías et al., 2016; Zioga et al., 2020; García-Valcárcel et al., 2022). Furthermore, the European Union and Great Britain have recently granted the emergency authorization for outdoor plant protection products containing the neonicotinoids clothianidin, imidacloprid and thiamethoxam in sugar beet (EFSA, 2021; www.gov.uk, 2021), thus highlighting the need for additional research to determine potential inadvertent side effects. While recent studies have shown that malnutrition and thiamethoxam can reduce worker HPG acini width (Minnameyer et al., 2021; Omar et al., 2017), effects on spermatozoa are only known for thiamethoxam exposure, but not for malnutrition (Minnameyer et al., 2021). In light of previous studies, we hypothesized that exposure to thiamethoxam and malnutrition individually will cause significant negative effects, likely resulting in increased stress (i.e., additive interactions) when combined.

2. Material and methods

2.1. Set-up: In a fully-crossed laboratory experiment, the single and combined effects of malnutrition and neonicotinoid exposure were tested on adult *Bombus terrestris* drones and worker females between May and June 2019. Newly emerged individuals (N=376; N_{drones} = 188; $N_{workers}$ = 188) of a defined age cohort (0-24 hours, based upon silvery-grey appearance (Alford, 1975) were collected from 50 colonies at Biobest Group NV (Westerlo, Belgium) and sent to the Institute of Bee Health within two days. Prior to being randomly assigned to a treatment group, the body mass (initial mass [mg]) of each bee was recorded to the nearest 0.1 mg using an analytic scale (Mettler Toledo AT400), and within-sex influence on measured traits (e.g. body size correlates with spermatozoa number in honey bee males, Schlüns et al., 2005). Treatments were as follows: 1. control (with pollen, N_{drones} = 64, $N_{workers}$ = 64), 2. malnutrition (pollen-deprived, N_{drones} = 32, $N_{workers}$ = 32), 3. neonicotinoid (with pollen and thiamethoxam, N_{drones} = 64, $N_{workers}$ = 64), or 4. combined (pollen-deprived and thiamethoxam, N_{drones} = 32, $N_{workers}$ = 32). No significant differences in initial mass were found amongst treatments (*bmct*: all *p's* = 1.00); however, drones (375.4 ± 4.64) were significantly larger than workers (247.04 ± 3.97, means [mg] ± std. error) by ~35%.

Bees were maintained in individual hoarding cages [100 cm^3] in incubators at 28 °C and 60% relative humidity in complete darkness (Minnameyer et al., 2021). To ensure sufficient carbohydrate intake and to expose bees to the neonicotinoid orally, individuals were provided with 50% [w/w] sucrose-solution *ad libitum,* via a syringe. Additionally, 1 g of neonicotinoid-free honey bee corbicular pollen (see Supporting Materials (SM) Document S1) was placed in the control and neonicotinoid treatment cages to provide protein for the

development of tissue and organs (Tasei and Aupinel, 2008). Mortality was recorded daily. All drones that survived the 12-day-exposure period were assessed for sperm traits at the Institute of Bee Health, whereas the worker heads were sent individually in separate 2 ml Eppendorf® tubes containing 0.5 ml of 2% paraformaldehyde PBS preservation buffer (Lanier and Warner, 1981) to the Department of Entomology and Plant Pathology, Auburn University, USA for hypopharyngeal gland (HPG) analysis and kept at 4°C until further processing.

2.2. Neonicotinoid solution preparation: Pure analytical grade thiamethoxam (Simga Aldrich 37924-100MG-R, UK) was dissolved in distilled water to produce a primary stock solution (1 mg ml^{-1}). It was then refrigerated at 4 °C in a tin-foil-covered flask to ensure complete darkness, thereby preventing potential UV-degradation of the active ingredient. Acetone was added as a dissolvent, accounting for < 0.5% of the volume in the final thiamethoxam sucrose solution. Every four days a fresh treatment solution was prepared by taking an aliquot of the primary stock solution to produce the desired concentration (20 ng ml^{-1}). While the chosen concentration is well beyond the average detected thiamethoxam residue levels in nectar (e.g., 0.05-3.2 ng g^{-1} (Botias et al., 2015; Zioga et al., 2020)), and thus likely reflects a 'worst case' scenario, similar higher concentrations have previously been reported in pollen and nectar of treated crops (e.g., (Stoner and Eitzer, 2012; Pilling et al., 2013)), as well as in herbaceous plants, wild flowers, guttation fluids or honey-dew (Calvo-Agudo et al., 2019; Wood et al., 2019; Hrynko et al., 2021; García-Valcárcel et al., 2022). To account for the potential negative effects of acetone, the dissolvent was also added to the control and malnutrition sucrose solutions.

2.3 Consumption and exposure: Sucrose-solution syringes were weighed and exchanged every four days to estimate daily [g day^{-1}] and total [g] consumption and to prevent possible fungal contamination (Williams et al., 2013). Multiplying consumption by the 20 ng ml^{-1} thiamethoxam concentration allowed for calculation of daily [ng day^{-1}] and total [ng] exposure per bee. Pollen consumption was measured by weighing the pollen ball to the nearest 0.1 mg at the start and end of the experiment. Consumption rates of individuals that did not survive the complete exposure period were excluded from the statistical analyses.

2.4. Sperm assessment: Spermatozoa count [thousands] (*N* = 123) and viability [%] (*N* = 128) were assessed for all drones surviving 12 days post-cage assay initiation (age = 14-15 days), when *B. terrestris* males are considered sexually mature (Tasei et al., 1998). Sperm assessments were performed following Straub et al. (2021b). In brief, the drones were anesthetized using CO_2, then pinned to a wax plate so that sperm samples could be collected (Baer and Schmid-Hempel, 2005). For this, the entire drone genitalia was placed in a 1.5 ml Eppendorf® tube containing 200 µl $Kiev^{+}$ buffer, and gently crushed to obtain a diluted sperm stock solution. Spermatozoa count and viability were then assessed to derive living spermatozoa quantity by multiplying the two factors (Minnameyer et al., 2021; Straub et al., 2021b). A detailed description of the sperm assessment method can be found in the supplementary materials (SM).

2.5. Hypopharyngeal gland assessment: Workers alive at the end of the experiment (day 12, age 14-15) were used for the HPG assessment (N = 114). Bumble bee HPGs are expected to be fully developed at this age and they remain the same size throughout their entire lifespan (Röseler, 1967). To obtain the HPGs, the heads were dissected (Carreck et al., 2013).

Then, single acini were accentuated by adding 0.5 ml of Coomassie Brilliant Blue g-250 stain (Hartfelder et al., 2013) and mounted on a wetted glass slide. Photographs of acini were taken with a digital microscope (Olympus DP72, Olympus, Tokyo, Japan) under a 5x compound light microscope (Olympus BX41, Olympus, Tokyo, Japan). Using ImageJ 1x, thirty acini diameters per bee were measured perpendicular to their attachment point, with 50 µm as a measurement scale (Schneider et al., 2012).

2.6. Statistical analyses: All analyses were conducted using STATA16 (StataCorp. 2019. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC.). Data were tested for normality with a Shapiro-Wilk's test and homogeneity of variances with the Levene's test and subsequent statistical methods were chosen accordingly. Individual bees represented the experimental units. Data for each response variable (i.e., pollen and sucrose consumption, sperm traits, and HPG acini width) were analyzed separately using a generalized linear (regression) model (GLM). Both treatments (i.e., malnutrition and neonicotinoid) and their interaction term (i.e., cross-product term, 'malnutrition#neonicotinoid'), were entered in the model as the fixed (explanatory) factors. Whenever direct comparisons between drones and workers were possible (e.g., sucrose consumption or survival), sex was added as an additional fixed factor to the model. The covariates body mass [g] and exposure [ng g^{-1}] were included in the models whenever appropriate. For each multiple regression model, a stepwise backward elimination approach was applied to determine the model of best fit. A goodness of fit for each model was assessed by the analysis of residuals, with the STATA function *predict [option deviance]*. Furthermore, best fit models were chosen by comparing every multi-level model with its single-level model counterpart using both a likelihood ratio (LR) test as well as the Akaike

information criterion (AIC) and the Bayesian information criterion (BIC), using the functions *lrtest* and *estat ic*, respectively (Sribney and StataCorp, 2005). If a model revealed a significant effect for either malnutrition or neonicotinoid exposure, it was followed up by a Bonferroni multiple pairwise comparisons test (*bmtc*) using the STATA function *mcompare*(*bonferroni*). Whenever appropriate, either the arithmetic means ± the standard error (SE) or medians ± 95% confidence intervals (CI) of non-transformed values, were reported in the text. All statistical figures were created using NCSS19 (NCSS version 19, Statistical Analysis Software, Kaysville, Utah, USA).

Survival time was set using the function *stset* and the *if* option was used for censored individuals. Differences in survival among treatments and between sexes were fitted using the *streg* function for survival models considering initial mass as the covariate (Leckie, 2010); data were plotted using Kaplan-Meier curves to visualize survival. Median longevity was calculated as the 50^{th} percentile of survival time (Lee and Wang, 2003). Individuals sampled on day 12 for sperm and HPG acini width assessments were right-censored for the survival analyses (Wei 1992).

Sucrose and pollen consumption, as well as HPG acini width and total living spermatozoa, were normally distributed (Shapiro-Wilk's test, $p > 0.05$) and modeled with a GLM using the function *glm,* where sex was added as an additional fixed factor and start mass as a covariate. As differences in pollen consumption were only compared between control and neonicotinoid treatment groups, the fixed factors malnutrition and the interaction term were removed from the model. Spermatozoa count at the individual drone level was non-normally distributed (Shapiro-Wilk's test, $p < 0.05$) and modeled with a GLM using the function *glm* and the option *family(gamma)*, where again start mass was used as a

covariate. Counter transforming the outcome variables to improve the linear correlation ceofficient, we opted for the Gamma family that provided good fits (normality of the residuals). Lastly, spermatozoa viability was considered as a score ranging from 0 to 100% with a subsequent multinomial distribution. Therefore, an ordered logistic regression model was applied using the function *ologit* (Greene, 2012), and start mass as a covariate.

2.7. Interactions: No interaction (i.e., neutral interaction) was assumed if the interaction term in the GLM was non-significant. However, if the interaction term revealed significance ($p < 0.05$), we then calculated the interaction effect size following Jackson et al. (2016) and Siviter et al. (2021). Here, the Hedges' *d* value, an estimate of the standardized mean difference not biased by small sample sizes, was used to calculate the interaction effect size. In brief, the interaction effect size was calculated as the standardized mean difference between the expected additive effect (i.e., stressor 1 effect + stressor 2 effect) and the actually observed effect (i.e., combined effect). Therefore, each interaction effect size was based on the absolute difference between the observed net impact of combined stressors against a hypothetical additive outcome based on the sum of their single independent effects (Jackson et al., 2016). The predicted additive effect (X_p) was calculated as:

$$X_P = (X_M - X_u) + (X_I - X_u) + X_u$$

where X is equal to the mean of each response variable from the four treatment groups (i.e., control (u), malnutrition (M), neonicotinoid insecticide (I)) and the X_p is equal to the predicted additive effect of both stressors M and I. Hedges' *d* effect sizes were then calculated for each observation by comparing the predicted additive effect (X_p) with the actual observed effect (X_o) of both stressors applied in combination, using the following equation:

$$Hedge's\ d = \frac{X_O - X_P}{S} * J$$

J represents a weighting factor based on the number of replicates (*n*), calculated as:

$$J = 1 - \frac{3}{4(n_0 + n_P - 2) - 1}$$

and *S* is the pooled standard deviation, calculated as:

$$S = \sqrt{\frac{(n_o - 1)\sigma_0^2 + (n_p - 1)\sigma_P^2)}{n_o + n_p - 2}}$$

where the predicted standard deviation σ_P^2 is calculated by pooling σ_M and σ_N. The pooled sample size n_p was calculated by adding the sample sizes of the two individual stressors (i.e., malnutrition and neonicotinoid insecticide) and n_o represents the number of samples in the combined treatment group. As stressors may impose both negative and positive effects on measured endpoint variables, we inverted the response direction/sign (+/-) for our calculated interaction effect sizes whenever the predicted additive effects (X_p) were negative (i.e., where both independent effects were negative) (Piggott et al., 2015). As a result, an interaction effect size (*d*) of zero represented an additive effect (i.e., a combined effect equal the sum of their independent effects), a positive *d* denotes a synergistic interaction (i.e., a combined effect greater than the sum of their independent effects) and a negative *d* reflects an antagonistic interaction (i.e., combined effect less than the sum of their independent effects).

3. Results

3.1. Survival, consumption, and exposure: Both malnutrition (z < 0.02, p = 0.86), neonicotinoid exposure (z = -0.89, p = 0.37) and the interaction term (z = -0.97, p = 0.33)

revealed a non-significant effect on survival (Table S1; Fig. S1). Likewise, no significant difference was observed between drones and workers (*z = -1.69, p = 0.091*), where the overall survival rate was 93.2 ± 90.69 – 95.8 % (cumulative survival [%] ± 95% CI). Furthermore, neither malnutrition (z = -1.06, p = 0.29) nor neonicotinoid exposure (z = 0.26, p = 0.80) had a significant effect on sucrose consumption (Fig. S2), where the interaction term also yielded non-significance (z = 0.34, p = 0.73; Table S1). Subsequently, thiamethoxam exposure between neonicotinoid and combined treatment groups did not significantly differ (*bmct*: p = 1.0). Likewise, pollen consumption did not significantly differ between the treatments that received pollen (i.e., controls and neonicotinoid) (z = -1.54, p = 0.13; Table S1, Fig. S3). However, sex-specific differences were observed for both sucrose and pollen consumption. Workers consumed 35% more pollen than the drones (z = 2.85, p = 0.004), whereas drones consumed 31% more sucrose-solution than workers (z = -0.91, p < 0.001), resulting in drones (66.24 ± 1.49 [ng]) being exposed to more thiamethoxam than workers (50.36 ± 1.54 [ng]; mean ± SE [ng]).

3.2 Sperm assessments: Both malnutrition (z = -3.63, p < 0.001) and neonicotinoid exposure (z = -2.72, p = 0.007) had a significant negative effect on spermatozoa count (Fig. 1A), as well as the interaction term (z = 2.00, p < 0.05; Table S1). Therefore, controls (611.74 ± 479.12 – 744.38) had significantly higher spermatozoa counts than all treatments (*bmct*: all *p's* < 0.004; Fig. 1A; median [thousands] ± 95% CI). However, the malnutrition (390.52 ± 300.27 – 480.76), neonicotinoid (322.51 ± 242.19 – 402.83), and combined (323.31 ± 247.13 – 399.50) treatment groups did not significantly differ from one another (*bmct*: all *p's* > 0.24; Fig. 1A; median [thousands] ± 95% CI). In comparison to controls, the reduction in spermatozoa count for the malnutrition, neonicotinoid, and combined

treatment groups was 36%, 47%, and 47%, respectively. Spermatozoa viability was only significantly negatively affected by neonicotinoid exposure (z = -4.60, p < 0.001), whereas malnutrition (z = 0.20, p = 0.94) and the interaction term (z = -1.03, p = 0.30) revealed non-significant (Table S1). Spermatozoa viability, therefore, did not significantly differ between control (86.13 ± 83.02 – 89.25) and malnutrition (86.56 ± 84.93 – 88.18) (*bmct*: p = 1.00; Fig. 1B); however, both were significantly higher compared to neonicotinoid (74.66 ± 70.99 – 78.34) and combined (73.41 ± 70.32 – 76.52) treatments (*bmct*: all *p's* < 0.001; Fig. 1B; median [%] ± 95% CI). The neonicotinoid and combined treatment groups did not significantly differ (*bmct*: all *p's* = 1.00 Fig. 1B) and were 13% and 15% less compared to controls, respectively. Similar to spermatozoa count, both malnutrition (z = -3.39, p = 0.001) and neonicotinoid exposure (z = -3.20, p = 0.001) had a significant negative effect on total living spermatozoa (Fig. 1C), and so did the interaction term (z = 2.17, p = 0.03; Table S1). Controls (514.62± 407.99 – 621.24) had significantly higher total living spermatozoa than the malnutrition (337.63 ± 259.87 – 415.38), neonicotinoid (244.26 ± 178.15 – 310.38), and combined (236.66 ± 181.66 – 291.66) treatment groups (*bmct*: all *p's* < 0.001; Fig. 1C; median [thousands] ± 95% CI). However, no significant differences were observed among the treatment groups (*bmct*: all *p's* > 0.14; Fig. 1C). In comparison to controls, the reduction in total living spermatozoa for the malnutrition, neonicotinoid, and combined treatment groups was 34%, 52%, and 54%, respectively.

3.3 Hypopharyngeal glands assessment: Malnutrition had a significantly negative effect on HPG acini width (z = -9.48, p < 0.001), whereas neonicotinoid exposure (z = -1.60, p = 0.11) and the interaction term (z = -0.25, p = 0.80) revealed non-significance (Table S1). Control

(54.74 ± 0.27) and neonicotinoid (54.22 ± 0.25) workers HPG acini width did not significantly differ (*bmct: p* = 0.65; mean ± SE [µm]; Fig. 2), yet both were significantly larger than the malnutrition (51.5 ± 0.22) and combined (50.81 ± 0.23) treatments (*bmct*: $p < 0.001$; mean ± SE [µm]; Fig. 2). The malnutrition and combined treatments did not significantly differ (*bmct; p* = 0.246; mean ± SE [µm]). In comparison to the controls, the reduction in HPG acini width for the malnutrition, neonicotinoid, and combined treatment groups was 7.5%, 2.7% and 8.8%, respectively.

3.4 *Interactions:* Interaction terms for sucrose consumption [g], survival [%], spermatozoa viability [%] and HPG acini width [µm] were all non-significant (all *p*-values > 0.30; Table S1), and therefore reflected a neutral interaction. In contrast, significant interactions were observed for spermatozoa counts [thousands] and total living spermatozoa [thousands] (both *p*-values < 0.05; Table S1). The calculated Hedges' *d* for spermatozoa count and total living spermatozoa was -0.79 and -0.62, respectively, revealing an antagonistic interaction.

4. Discussion

Our data support that the impact of stressor interactions need to be regarded in context and should not be considered as being additive or synergistic *per se* - as the narrative of past studies often implies. Individual exposure to malnutrition and thiamethoxam led to similar sublethal negative effects on sperm traits and HPGs in bumble bees. Likewise, the combined exposure treatment revealed negative sublethal effects which were similar to those observed in the individual stressor groups, resulting in either neutral (i.e., survival, consumption, spermatozoa viability and HPG acini width) or antagonistic (i.e., spermatozoa counts and total living spermatozoa) interactions. . Our data suggest that key fitness traits may be under strong selection and likely buffered against stochastic environmental

stressors. To improve future forecasts of environmental change and safeguard biodiversity, it is important to identify prevailing stressors and quantify the actual nature of their interactions to successfully mitigate potential negative effects on insect health.

Detoxification deficiency due to malnutrition and starvation can alter a bees' ability to metabolize and tolerate xenobiotics (Berenbaum and Johnson, 2015; Gong and Diao, 2017). For instance, bees consuming high-quality pollen displayed greater pesticide resistance compared to bees consuming pollen of inferior quality (e.g., low protein levels) (Wahl and Ulm, 1983). This could be explained by a deficiency of essential amino acids that are key carrier substances in the detoxification pathway (du Rand et al., 2015). In response to the lack of certain nutrients, individuals may compensate by increasing their consumption behaviour of other available nutrients (Castañeda et al., 2009). Compensation for the lack of nutrition by consuming more sucrose-solution of pollen-deprived individuals (Vaudo et al., 2017) and reduction in pollen consumption (Laycock et al., 2012) as previously observed is unlikely, as the treatment in our study revealed no significant effect on consumption behavior. This may be due to differences in chemical substances (i.e., thiamethoxam (this study) vs. imidacloprid (Vaudo et al., 2017)) as well as cage assay conditions (i.e., individual hoarding (this study) vs. micro-colonies (Laycock et al., 2012)). However, workers consumed more pollen yet less sucrose-solution than their male counterparts. While pollen consumption likely varies between workers and drones due to division of labor within the colony (Szolderits and Crailsheim, 1993), the increased consumption of sucrose-solution by the drones is most likely linked to varying body size and/or metabolic rates (Heinrich, 1972). Subsequently, drones were exposed to higher dosages of thiamethoxam than workers, which emphasizes the urgency to account for sex-specific differences during pesticide risk

assessments (Friedli et al., 2020). Despite drones being exposed to 32% higher dosages of thiamethoxam, sex-specific differences in mortality were not observed, where mortality rates across all treatments were very low. While our data confirm previous laboratory findings that short-term field-realistic exposure to thiamethoxam does not reduce individual bumble bee survival (Baron et al., 2017; Straub et al., 2021a), they deviate from a previous study showing negative effects of pollen deprivation on bumble bee worker survival (Smeets and Duchateau, 2003). This may be due to the varying experimental conditions (see above), as well as the monitoring duration of survival (i.e., 10 days v. two months). Future efforts to standardize methodology would make comparison among studies easier (Carreck et al., 2020). It remains to be tested if effects of malnutrition and thiamethoxam exposure on bumble bee survival may have become apparent if measured for a longer duration, at a higher level of exposure or under field conditions, as found in other studies (Minnameyer et al., 2021; Mommaerts et al., 2010; Straub et al., 2021b). Regardless, the data support that short-term neonicotinoid exposure to adults may not have lethal consequences, but rather imposes negative sublethal effects, such as on key fitness parameters (e.g., colony development and production of sexuals, or sperm traits (Minnameyer et al., 2021; Whitehorn et al., 2012). Future studies should investigate potential trade-offs between longevity and other important physiological traits in bees, which are well known for other organisms (Edward & Chapman, 2011; Flatt and Heyland, 2011) and are most likely the consequence of functioning, yet costly, detoxification mechanisms (Tyler et al., 2006; Strobl et al., 2020). Regardless of the mechanism, the incorporation of additional key fitness-related sublethal endpoint variables (e.g., sperm traits), besides survival, appear long overdue to ensure robust and reliable pesticide evaluations as survival alone may be misleading and may lead to flawed risk assessments (Straub et al., 2020b).

Our data show for the first time that pollen-deprivation of adult males can reduce spermatozoa counts in bumble bees, which is in line with earlier data for other social insects (Pech-May et al., 2012; Dávila and Aron, 2017) and solitary ones (Gage and Cook, 1994; Bunning et al., 2015). As spermatogenesis and spermiogenesis are completed upon adult emergence in all bees (Hoage and Kessel, 1968), it appears plausible that a lack of essential amino acids may affect sperm migration from the testes to the seminal vesicles. While little is known for male bees, muscular contractions are likely responsible for the passive translocation of spermatozoa within the male insect genitalia (Werner and Simmons, 2008). Such 'sperm pumps' are well known in male Diptera, Strepsiptera or Antliophora (Hexapoda) species (Hünefeld and Beutel, 2005) and similar muscular tissues/structures have been described along the female honey bee and bumble bee spermathecal duct (Bresslau, 1905; Schoeters and Billen, 2000). Therefore, as in the maturation of honey bee flight muscles (Hendriksma et al., 2019), pollen-deprivation and the subsequent lack of essential amino acids may negatively affect the development and/or functioning of such muscular tissue in the adult male genitalia, thereby explaining the reduced spermatozoa counts compared to the controls. Likewise, thiamethoxam exposure resulted in reduced spermatozoa counts compared to controls. As neonicotinoids act on the nervous system and can cause neuronal hyper-excitation (Belzunces et al., 2012), thiamethoxam may have caused a neuronal dysfunction of muscles underlying the transport of the spermatozoa from the testes to the vesiculae seminales (e.g., sperm pump). The reduced spermatozoa counts in the spermathecae of neonicotinoid-exposed honey bee queens, *Apis mellifera* (Williams et al., 2015) may also be explained by such neurological dysfunction. During mating of *A. mellifera*, sperm is first transferred to the oviducts and then secondarily transported to the spematheca via the Bresslau sperm pump (Bresslau, 1905). As bumble bees, *Bombus* spp.,

are characterized by multiple mating of males and single mating of females (Paxton, 2005; please refer to Brown et al., 2002 for the naturally polyandrous bumble bee, *Bombus hypnorum* as a notable exception), such a striking 40% reduction in male spermatozoa counts due to either single exposure scenario will very likely reduce both male fitness (Kraus et al., 2004) as well as overall colony fitness. Indeed, fewer spermatozoa will jeopardize chances of successfully inseminating multiple females. Further, due to haplodiploidy in the hymenoptera and complementary sex determination (Beye et al., 2003), male genes are only transferred to the next generation via production of diploid female offspring (mating with rare diploid males result in *Bombus* colony failure (Duchateau and Mariën, 1995)). Further, due to the reproductive division of labor in the social hymenoptera, colony success strongly depends on the intitial production of female worker offspring. Therefore, given that queens cannot successfully inseminate an egg despite release of sperm during oviposition, such erroneously produced males may seal the fate of the entire colony. It appears as if males with few spermatozoa can have drastic effects on entire *Bombus* populations since the possible number of fertilized eggs by any given queen determines size and fitness of colonies.

Our data are in line with earlier work suggesting that pollen consumption by adult bees likely does not promote spermatozoa viability (Stürup et al., 2013). Indeed, spermatozoa viability may not be affected by pollen/protein consumption during the bees' adult life because seminal fluid proteins, important components to maintain sperm viability, are already produced during the bees' pupal development (Avila et al., 2011). Alternatively, other molecules of the seminal fluid, such as salts, vitamins, lipids or vitamins may affect the seminal fluid and sperm viability in adult bees (Scolari et al., 2021). The functional role of

these molecules is not well understood in adult insect males, particularly if malnutrition and / or neonicotinoid exposure can affect seminal fluid quality and potentially sperm viability. Irrespective, the results may also suggest a stronger selective pressure on sperm viability than on high numbers of spermatozoa. The rationale behind this being that males' spermatozoa numbers are typically not a limiting factor for a successful mating, while the percentage of fertilization-competent spermatozoa transferred to the females (gynes) is a key element of fitness for both sexual partners. Therefore, the observed reduction in spermatozoa viability due to exposure to thiamethoxam is of considerable concern, and is in line with previous evidence suggesting neonicotinoids can adversely impactmale insect fecundity (Minnameyer et al., 2021; Straub et al., 2021b, 2016). The observed reduced spermatozoa viability may be a result of neonicotinoid exposure impairing spermatozoa mitochondria or seminal fluid proteins which as a result may have increased oxidative stress in spermatozoa (Ciereszko et al. 2017; Abdelkader et al., 2019). Alternatively, yet not mutually exclusive, the observed effects may be directly associated to thiamethoxam's mode of action (i.e., binding to the acetylcholine site of the nicotinic acetylcholine receptor (nAChR) (Matsuda et al., 2021). Additional studies are required to understand the precise mechanisms responsible for how thiamethoxam exposure interferes with bumble bee male reproductive physiology.

Our results confirm previous studies in honey bees that malnutrition can negatively affect the HPG acini width (Crailsheim and Stolberg, 1989; Pernal and Currie, 2000). The observed negative effect of pollen-deprivation is likely due to the lack of essential amino acids required for adequate gland development and growth (Crailsheim, 1990; Hendriksma et al., 2019). Reduced HPG acini width may adversely affect collaborative brood care by reducing

worker nursing abilities, which may lead to precocious shifts from nursing to foraging duties (Crall et al., 2018). Such downstream effects will have negative consequences for colony development as well as the production of sexuals (i.e., drones and gynes). Control acini width (~55 µm) was comparatively smaller than reported in a previous study (~60 µm, (Albert et al., 2014)), likely attributed to the absence of brood during our work (DeGrandi-Hoffman et al., 2010). Further, the data revealed that thiamethoxam exposure did not significantly affect HPG acini width when compared to controls. This finding deviates from previous studies and may be explained by the different choice of chemical substances (i.e., thiamethoxam vs imidacloprid (Hatjina et al., 2013)), varying thiamethoxam concentrations (i.e., 20 ng g^{-1} vs 40 ng g^{-1} (Renzi et al., 2016)), as well as bee species (i.e., bumble bees vs. honey bees (Hatjina et al., 2013; Renzi et al., 2016)). However, our data confirm that an adequate insect diet can either reduce an individual's sensitivity towards xenobiotic stress or increase their tolerance to insecticides by stimulating the production of detoxification enzymes (Terriere, 1984; Deans et al., 2017; Barascou et al., 2021;). This is evidenced when comparing our data to Minnameyer et al. (2021), which revealed significant negative effects of thiamethoxam exposure of feeding glands when *B. terrestris* individuals were deprived of pollen.

While the lack of proteins can have substantial negative effects on the quality and development of muscles in bees, and the neurotoxic effects of neonicotinoids are known to impair the functioning of muscles, one would expect that the combined exposure should yield harmful additive effects. However, surprisingly, we found the contrary (i.e., neutral or antagonistic interactions). The observed neutral interactions are likely explained by either only one (i.e., spermatozoa viability and acini width) or neither (e.g., survival and

consumption) of the stressors imposing a significant effect on the measured variable. The exact mechanism responsible for the observed antagonistic interactions is not known, yet previous studies in insects suggest that they are indeed far more common than often presumed (Côté et al., 2016; Bird et al., 2021; Schläppi et al., 2021). One plausible explanation for the antagonism may be explained by the common phenomena of pesticide-induced hormesis (Guedes et al., 2022). Hormesis is a biphasic dose-response whereby exposure to low levels of pesticides can stimulate biological processes such as reproductive output or other fitness relevant traits in arthropods including aphids, crickets, and mites (Guedes and Cutler, 2013; Rix and Cutler, 2020). However, the awareness of such hermetic effects is currently limited in bee research, despite the increased emphasis of toxicology (Cutler and Rix, 2015). A speculative explanation as to why antagonistic interactions were revealed in spermatozoa counts and total living spermatozoa may be linked to the fundamental importance of these components for male fitness. The inability to transport spermatozoa from the testes to the seminal vesicles would result in infertile males, and subsequently reflect the worst possible fitness constraint. It therefore appears likely that such a core trait of male fitness is under strong selection to buffer against potential malfunctioning at all costs. If this is the case, muscles responsible for the migration of spermatozoa to the seminal vesicles could have a higher resilience threshold to ensure functionality. Given trade-offs occur, such fitness relevant traits may be maintained at the expense of other non-investigated traits. Thus, additional empirical data are urgently required to better understand the processes and mechanisms of single stressors, before being able to predict the complex nature of stressor interactions. Only then, can we improve our understanding of how interaction effects may occur, as well as how they may vary

among environmental stressors (Siviter et al., 2021) and within and between different bee species (Straub et al., 2020a).

5. Conclusion

Due to the widespread contamination of neonicotinoid insecticides (Mitchell et al., 2017) as well as other pollutants (de Souza Machado et al., 2018; Tang et al., 2021) and the increased loss of nutritional diversity due to habitat loss (Carvell et al., 2006), our data provide additional plausible mechanistic explanations for reduced bumble bee colony growth and reproduction under field conditions (Rundlöf et al., 2015). It is important to note, however, that our laboratory findings are potentially an underestimation of the negative effects that can be expected under field conditions, as both malnutrition and neonicotinoid exposure are known to impair bumble bee larvae (Moerman et al., 2017; Leza et al., 2018). This, as well as increased flight distances due to inadequate forage availability or poor weather periods may further enhance the observed negative effects. Future studies at the colony level and ideally under field conditions are necessary to investigate potential adverse effects on key fitness components such as sperm traits and HPG acini width when exposed at different life-history stages (Van Oystaeyen et al., 2020). Nevertheless, our study provides clear evidence that multiple stressor interactions must be considered within context, and not automatically assumed to be additive or synergistic as commonly reported. Moreover, combined exposure effects can vary depending on the measured endpoint and level of evalution (Schläppi et al., 2021), warranting additional studies to elucidate the complex consequences of concurrent environmental exposure to bee species (Siviter et al., 2021). It appears long overdue that regulatory authorities incorporate the evaluation of combined stressor interactions into current environmental risk assessments (Topping et al., 2020). This

would not only improve our understanding of how stressors interact, but also reflect a more field-realistic scenario and enable policy-makers to implement adequate and sustainable measures to safeguard biodiversity. Lastly, to prevent further unsustainable damage to ecosystems, policy-makers should strengthen the implementation of integrated pest management (Wyckhuys et al., 2021) and take immediate action to overhaul the prophylactic usage of pesticides for agricultural purposes. Furthermore, to mitigate negative impacts of the decline in availability and diversity of nutrients, conservation efforts to protect semi-natural habitats as well as restoration of floral resources should be promoted to effectively promote and protect wild insect populations (Scheper et al., 2013; Ganser et al., 2021).

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Figure and table captions:

Figure 1. Bumble bee (*Bombus terrestris*) drone sperm assessment. Assessment of sperm traits in drones under malnutrition (N = 32), neonicotinoid insecticide (N = 64) and combined (N = 32) exposure compared to controls (N = 64). (**A**) Comparison of spermatozoa counts showed a significant difference between controls and treatment groups (Bonferroni multiple-pairwise comparison test (*bmct*); $p < 0.05$). (**B**) Percentage of viable spermatozoa revealed no significant difference between controls and malnutrition (*bmct*: $p > 0.05$); however, both treatments significantly differed from the neonicotinoid insecticide and combined treatment groups (*bmct*: $p < 0.05$). (**C**) Control total living spermatozoa significantly differed from all treatment groups (*bmct;* $p < 0.05$), yet no significant difference was observed among the treatment groups (*bmct*: $p > 0.05$). All bar charts show medians and 95% Confidence Intervals; dots represent individual measurements. Significant differences among treatment groups are indicated by different capital letters (*bmct*: $p < 0.05$).

Figure 2. Bumble bee (*Bombus terrestris*) worker hypopharyngeal gland (HPG) acini width assessment. Assessment of HPG acini width in workers under malnutrition (N = 32), neonicotinoid insecticide (N = 64), and combined (N = 32) exposure compared to controls (N = 64). HPG acini width revealed no significant difference between controls and neonicotinoid insecticide (Bonferroni multiple-pairwise comparison test (*bmct*); $p > 0.05$); however, both treatments significantly differed from the malnutrition and combined treatment groups (*bmct;* $p < 0.05$). All bar charts show medians and 95% Confidence Intervals; dots represent individual measurements. Significant differences among treatment groups are indicated by different capital letters (*bmct*: $p < 0.05$).

Table 1. Summary of sample sizes and results of the effects of neonicotinoid insecticide and malnutrition individually and in combination for all outcome variables measured on male and female worker bumble bees (*Bombus terrestris*).

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Outcome variable	Sex	Treatments	Sample size	Mean	Standard error	Median	95% Confidence intervals	
							Lower	Upper
Cumulative survival [%]	Females and males	Control	124			92.74	88.18	97.31
		Malnutrition	64			93.75	87.82	99.69
		Neonicotinoid	124			96.77	93.66	99.88
		Combined	64			98.44	95.4	100
Total sucrose consumption [g]	Female	Control	57	2.44	0.69			
		Malnutrition	31	2.41	0.47			
		Neonicotinoid	58	2.57	0.67			
		Combined	32	2.38	0.68			
	Male	Control	56	3.54	0.49			
		Malnutrition	30	3.48	0.71			
		Neonicotinoid	55	3.43	0.61			
		Combined	31	3.52	0.59			
Pollen consumption [g]	Female	Control	56	0.167	0.08			
		Malnutrition						
		Neonicotinoid	58	0.164	0.07			
		Combined						
	Male	Control	51	0.122	0.05			
		Malnutrition						
		Neonicotinoid	53	0.101	0.0			
		Combined						
Sperm counts [thousands]	Male	Control	32			611.74	497.12	744.8
		Malnutrition	30			390.52	300.27	480.76
		Neonicotinoid	37			322.51	242.19	402.83
		Combined	30			323.31	247.13	480.76
Sperm viability [%]		Control	32			86.13	83.03	89.25
		Malnutrition	30			86.56	84.84	88.18
		Neonicotinoid	37			74.67	70.99	78.34
		Combined	30			73.42	70.31	76.52
Total living sperm [thousands]		Control	32			514.62	407.99	621.24
		Malnutrition	30			337.63	259.87	415.38
		Neonicotinoid	37			244.26	178.15	310.38
		Combined	30			236.66	181.66	291.66
Hypophyrangeal gland length [µl]	Female	Control	490	54.77	0.27			
		Malnutrition	547	51.53	0.22			
		Neonicotinoid	549	54.22	0.25			
		Combined	600	50.8	0.23			

Table 1

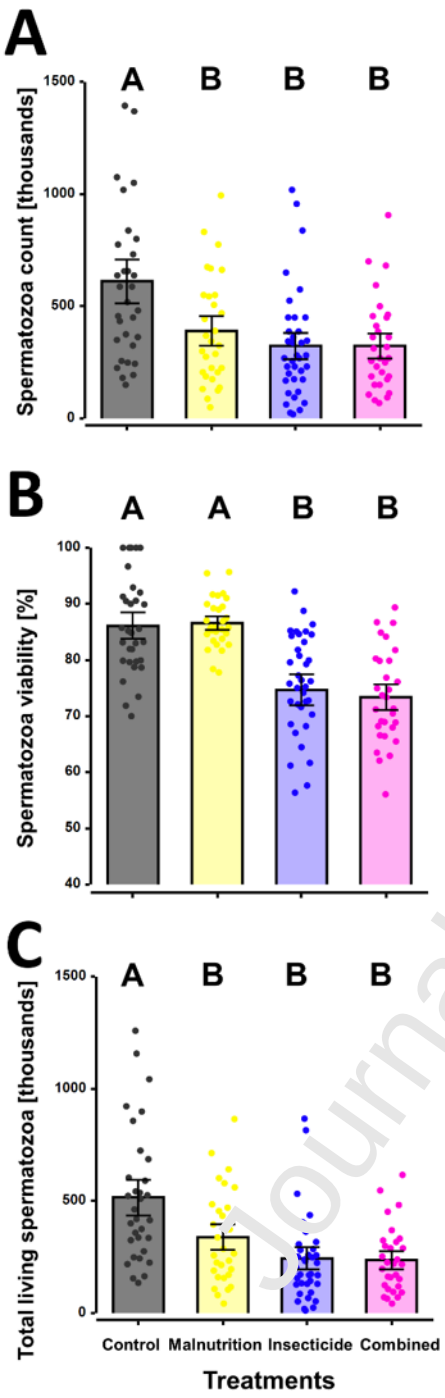


Figure 1

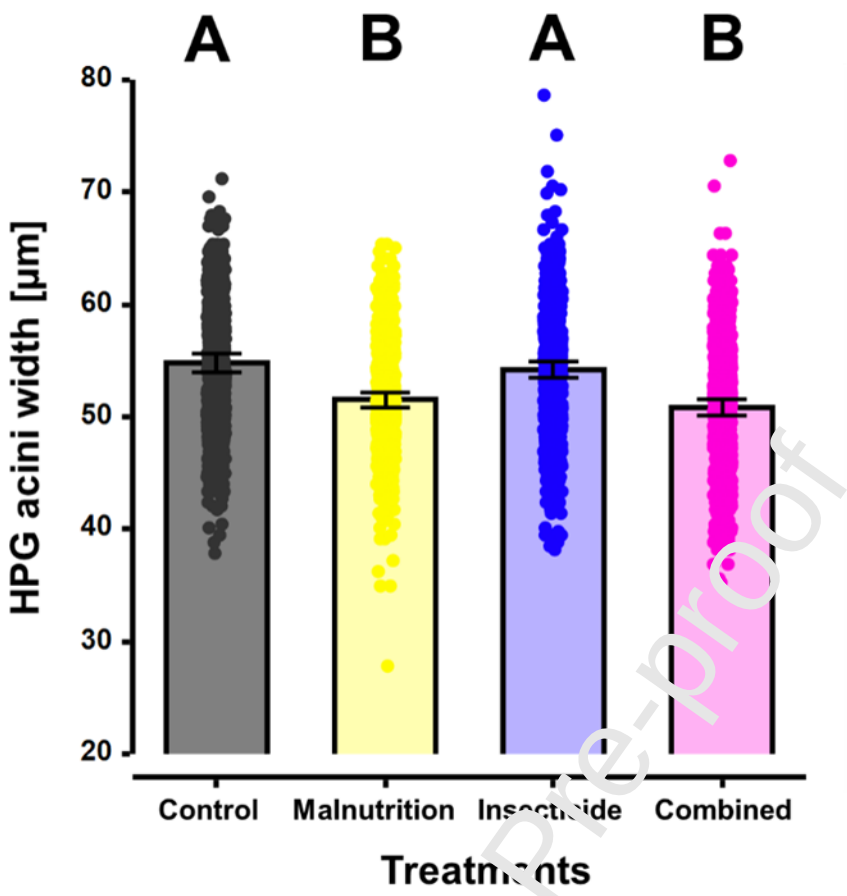
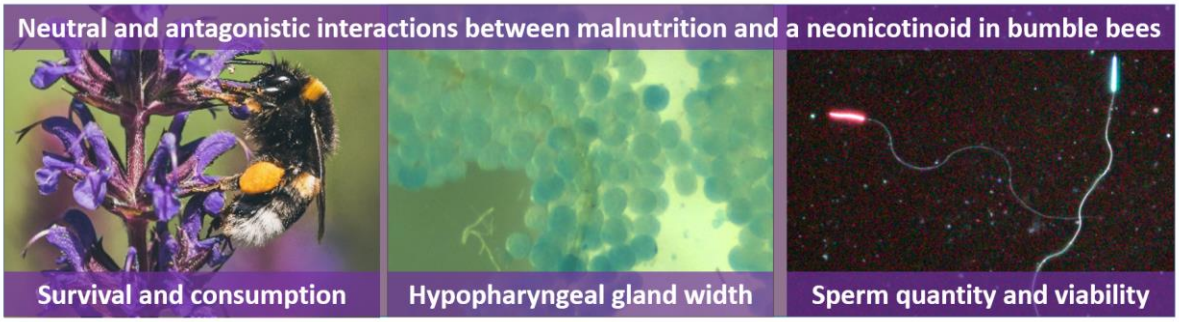


Figure 2



Graphical Abstract

Highlights

- Interactions between environmental stressors can reduce insect fitness
- Malnutrition and an insecticide negatively affected fitness traits in bumble bees
- Combined exposure resulted in either neutral or antagonistic interactions
- The findings may be due to key fitness traits being resilient to stressor exposure
- Further empirical tests are required to understand stressor interactions in bees