Toxicology Reports 9 (2022) 36–45

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Thiamethoxam as an inadvertent anti-aphrodisiac in male bees

Lars Straub [a,b,*], Angela Minnameyer [a], Domenic Camenzind [a], Isabelle Kalbermatten [a], Simone Tosi [c], Annette Van Oystaeyen [d], Felix Wäckers [d], Peter Neumann [a,b], Verena Strobl [a]

[a] Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[b] Agroscope, Swiss Bee Research Centre, Bern, Switzerland
[c] Department of Agricultural, Forest, and Food Sciences, University of Turin, Italy
[d] Biobest Group NV, Westerlo, Belgium

ARTICLE INFO

Handling Editor: Dr. Aristidis Tsatsakis

Keywords:
Bombus terrestris
Drone
Gyne
Mating
Neonicotinoid insecticide
Sperm

ABSTRACT

Sexual reproduction is common to almost all multi-cellular organisms and can be compromised by environmental pollution, thereby affecting entire populations. Even though there is consensus that neonicotinoid insecticides can impact non-target animal fertility, their possible impact on male mating success is currently unknown in bees. Here, we show that sublethal exposure to a neonicotinoid significantly reduces both mating success and sperm traits of male bumblebees. Sexually mature male *Bombus terrestris* exposed to a field-realistic concentration of thiamethoxam (20 ng g^{-1}) or not (controls) were mated with virgin gynes in the laboratory. The results confirm sublethal negative effects of thiamethoxam on sperm quantity and viability. While the latency to mate was reduced, mating success was significantly impaired in thiamethoxam-exposed males by 32% probably due to female choice. Gynes mated by exposed males revealed impaired sperm traits compared to their respective controls, which may lead to severe constraints for colony fitness. Our laboratory findings demonstrate for the first time that neonicotinoid insecticides can negatively affect male mating success in bees. Given that holds true for the field, this provides a plausible mechanism contributing to declines of wild bee populations globally. The widespread prophylactic use of neonicotinoids may therefore have previously overlooked inadvertent anti-aphrodisiac effects on non-target animals, thereby limiting conservation efforts.

1. Introduction

Sexual reproduction is essential in almost all multi-cellular organisms [1]. Therefore, factors jeopardizing sexual reproduction can have profound effects on an individual's fitness, which may severely affect entire populations [2]. Proximate factors that can influence reproductive success include seasonality [3], diet [4], climate change [2], predation [5], as well as the exposure to environmental pollution [6]. It is evident that ubiquitous environmental pollutants are taking their toll on both humans as well as the animal kingdom in a vast and far-reaching manner [7,8]. The unleashing of such pollutants into our environment are of particular concern if they endanger sexual reproduction, thereby possibly threatening the very existence of a species [6].

Increasing reports suggest that environmental pollutants are adversely affecting reproductive functions in humans as well as in animals [9]. A fertility crisis, as reported in men [10], has been closely associated with the increasing abundance of industrial pollutants (i.e., plastics and pesticides) in our environment [11,12]. Likewise, such toxicants have revealed adverse effects on animal fertility by negatively impairing reproductive morphology and physiology [13,14], libido [15], as well as sexual behaviour and reproductive success in males [16]. In light of their significant role for sexual reproduction, it is not surprising that such advert effects on male reproductive health can result in marked declines in populations, as recently observed in seals, alligators, fish and birds [17]. With respect to the global rising levels of persistent chemical toxicants across ecosystems [18], it appears obvious that negative effects on reproductive success and fertility offer a plausible mechanistic explanation for recent declines in wildlife populations. Particularly worrisome are recent global insect declines as their roles in maintaining ecosystem functioning and human food security are essential [19].

Being amongst one of the most frequently applied insecticides globally [20], neonicotinoid insecticides have received considerable attention, as they are argued to act as a driver of widespread declines in

* Corresponding author at: Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland.
E-mail address: lars.straub@vetsuisse.unibe.ch (L. Straub).

https://doi.org/10.1016/j.toxrep.2021.12.003
Received 6 September 2021; Received in revised form 3 December 2021; Accepted 4 December 2021
Available online 8 December 2021
2214-7500/© 2021 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

insect pollinator diversity and abundance [21,22]. Due to their systemic nature to translocate and persist within plant tissues as well as disseminating into soil and appearing in groundwater [23], pollinating insect species are likely to encounter exposure via consumption of pollen, nectar, guttation fluids as well as water - even months after the insecticide was applied [24,25]. Neonicotinoid insecticides act as agonists of the postsynaptic nicotinic acetylcholine receptors and are considered highly toxic for insects even at low concentrations [26], causing neurotoxic effects that can result in death [23]. Moreover, these neurotoxins are known to elicit a wide array of sublethal effects on pollinating bees, such as inducing hyper- or hypoactivity, which can impair flight and feeding behaviour [27,28]. Furthermore, neonicotinoid exposure has been shown to reduce offspring production [29,30] and impair reproductive physiology in females and males by reducing ovary development and sperm capacities [31–33]. However, to our knowledge, no data exist on the effects on male bee mating behaviour and mating success in combination with fertility, despite such effects being shown in other arthropod species. For instance, parasitic wasps revealed an impaired ability to detect sex pheromones and mating cues [34,35]. Further, spiders and moths showed reduced courtship behaviour due to reduced contact chemoreception functioning and mating calling [36, 37], respectively. Given such effects hold true for bee species, combined with negative effects on sperm traits as previously observed [32], this may have drastic consequences for entire populations.

Neonicotinoids have been implicated as a factor contributing to the mounting evidence of increasing insect population declines and local species extinctions, to which bumblebees are no exemption [38]. Bumblebees constitute an ideal model organism to study potential effects of toxicants on individual fitness and fertility due to the high probability of being exposed as well as due to their mating behaviour and life-history. *Bombus* males (drones) show various mating behaviours (e.g., territoriality, scent-marking and patrolling, or nest surveillance) in hopes of finding a virgin queen (gyne) to copulate with [39]. Copulation generally lasts between 10–80 minutes and is imposed upon the females by the males to ensure that their sperm has at least partially transferred from the female oviducts to the spermatheca as well as the formation of a mating plug that reduces female receptiveness [39]. With the exception of *Bombus hypnorum* [40], bumblebee queens rely on a single mating event (monandry), whereas the males can mate multiple females (polygyny) [39]. Subsequently, a single drone must be able to provide sufficient sperm to fill the spermatheca of one or multiple gynes and so provide them with a lifetime sperm supply. This is further underlined by the inefficient sperm transfer during copulation leading to a surplus of sperm found in gynes *bursa copulatrix* that does not reach the spermatheca [41]. Therefore, increased sperm quantity will increase male fitness, as it will enable to successfully inseminate more females. More importantly, viable sperm is essential to ensure fertilization of eggs in order to produce female offspring. This is key for male fitness in *Bombus* as the drone offspring of mated gynes is produced via parthenogenesis (arrhenotoky) and therefore not related to their male partners. However, the post-mating sperm-transfer mechanism in *Bombus* gynes is not as well-known as in honeybees, *Apis* ssp. In honeybees, the queens copulate with many males on a single or multiple mating flights, and the semen of the drones is initially stored in the lateral oviducts [42]. Then, the semen of all males is simultaneously transferred into the spermatheca, not only by active migration, but also passively via a sperm pump [43]. However, only a small proportion of the sperm essentially reaches the spermatheca (between 5% and 10%), while the rest is ejected via the sting chamber [44]. Given that holds true for *Bombus*, impaired sperm viability may decrease the quantity of living sperm reaching the spermatheca. Moreover, increased non-viable sperm stored in the spermatheca may lead to an unintentional male-biased offspring sex ratio and a reduction in female worker force due to a lower probability of egg fertilization. This may result in smaller colonies and subsequently leading to a reduced production of gynes [39]. While effects of neonicotinoid exposure on females (i.e., gynes, queens and workers) and colony fitness have been reported [29,45,46], effects on male mating behaviour and sperm traits inside the spermatheca remain unknown in bumblebees.

Here, we tested the effects of a commonly applied neonicotinoid insecticide on the mating behaviour and fertility as tokens of fitness of male bumblebees, *Bombus terrestris*. For that purpose, we measured the effects of field-realistic thiamethoxam exposure on male survival, consumption, mating behaviour and reproductive physiology (i.e., sperm quantity and viability). In addition, we investigated sperm quantity and viability in the females' spermatheca shortly after mating to determine whether sperm was further impaired during the copulation and insemination process. In light of previous studies [33], we hypothesize that thiamethoxam would negatively affect male mating success and fertility, as well as consequently compromising female reproductive physiology.

2. Methods

2.1. Experimental set-up and insecticide solution preparation

The experiment was conducted between August and October 2019 at the Institute of Bee Health, University of Bern, Switzerland. To establish known age-cohorts, newly emerged *Bombus terrestris* drones ($N = 140$) were randomly selected from various colonies at Biobest Group NV, Westerlo, Belgium, based upon their physical appearance [47]. Due to shipping duration to Switzerland, the bees were between two and three days of age upon arrival. Before drones were randomly assigned to either neonicotinoid insecticide or control treatment groups, their start body mass (hereafter, start mass) was recorded to the nearest 0.1 mg using an analytical scale (AT400, Mettler Toeldo, Ohio, USA). Each treatment consisted of 62 drones that were maintained individually in standard hoarding cages [100 cm^3] and held in incubators at 25 °C and 60% RH in complete darkness [33].

Each drone was provided with 50% [w/w] sucrose-solution *ad libitum* via a 5 ml syringe to provide sufficient carbohydrates. Sucrose-solution for neonicotinoid-exposed drones additionally contained 20 ng g^{-1} thiamethoxam. While this concentration may be above the average thiamethoxam residue levels detected (e.g., 3.2 ng g^{-1} [48]), such similar higher concentrations have indeed been reported in nectar and pollen of treated maize [49] and squash [50], as well as in herbaceous plants [51], wild flowers [48], guttation fluids [52], and honeydew [53]. In addition, *B. terrestris* males are known to fly up to 60 km a day when patrolling for virgin gynes as well as foraging [39], resulting in a high energy demand. Therefore, daily consumptions rates are most certainly higher than those observed under laboratory conditions. Subsequently, drones under natural conditions may experience similar chronic exposure rates as in our study, despite field residue levels being potentially lower. To obtain the desired thiamethoxam solution, pure thiamethoxam (99% purity, 37924-100MG-R, CAS-Number: 153719-23-4; Sigma-Aldrich®, Buchs, Switzerland) was dissolved in distilled water and acetone at a nominal concentration of 1000 mg L^{-1}, which was then diluted to a secondary stock solution [1 mg L^{-1}]. A known volume of the secondary solution was then used to dose the sucrose-solution of the neonicotinoid treatment group. Acetone accounted for less than 0.5% of the volume in the final thiamethoxam sucrose-solutions and was added to ensure complete thiamethoxam dilution. The control sucrose-solution contained the identical amount of acetone. Syringes were weighed and replaced every four days with newly prepared sucrose-solutions to prevent possible fungus contamination as well as to limit insecticide degradation. Further, 1 g of pesticide-free corbicular honeybee pollen formed into a small pellet was provided in each cage (see supplementary information (SI) Fig. 1A) to ensure each bee had sufficient protein resources for organ and tissue development [54]. Drones were exposed to their respective treatments for a total of 12 days. Individuals that have survived the exposure period were then used to assess mating behaviour and/or sperm traits.

2.2. Pollen and sucrose consumption, exposure, survival, body mass difference

Sucrose consumption was measured by recording the mass of the syringe before and after every four days until the experiment was terminated or at the point of an individual's death. This enabled to calculate the absolute thiamethoxam-exposure rates for each drone. Pollen consumption was measured by subtracting the mass of the provided pollen pellet at either day 12 or at the point of death from the initial start mass. To account for evaporation, three cages containing a sucrose-solution feeder and a pollen pellet without a bee were included as evaporation controls. Evaporation for both sucrose-solution and pollen feeding systems were below 1% after 12 days and thus considered negligible. Exposure rates were calculated by multiplying the mass of consumed sucrose-solution [g] by the concentration of thiamethoxam [ng g^{-1}]. Survival was recorded daily, whereby drones surviving the exposure period were aged between 14 and 15 days, and thus considered sexually mature [55]. All surviving sexually mature drones were again weighed as previously described to obtain a post-exposure mass (hereafter, end mass). To determine the body mass difference, the end mass was subtracted from the start mass of each individual.

2.3. Mating behaviour

A subset of drones aged between 14 and 15 days was randomly selected for a mating behaviour trial from the control ($N = 23$) and neonicotinoid treatment ($N = 26$). The remaining drones from either treatment ($N_{control} = 39$; $N_{neonicotinoid} = 36$) were directly used for sperm assessments (i.e., quantity and viability). Newly emerged virgin bumblebee gynes ($N = 70$) were obtained from the Biobest Group NV (Belgium) in a transparent plastic box [15 × 15 × 10 cm]. Upon arrival, the gynes remained together and were kept in an incubator at 25 °C and 60% RH and complete darkness. The gynes were provided with *ad libitum* access to pesticide-free corbicular honeybee pollen and 50% [w/w] sucrose-solution. The gynes were held under these conditions for six days; thereafter all gynes were between 8–9 days of age and considered sexually mature and receptive [39]. Then, gynes were placed into individual mating arenas [250 cm^3] (SI Fig. 1B; SI Fig. 2) at room temperature (~25 °C) and exposed to natural light. Mating couples were paired by randomly assigning a male from either control or insecticide treatment to a mating arena.

Following Amin et al. [56], three mating behaviours were recorded: mating latency (i.e., the time between the introduction of the drone into the mating arena until initiation of copulation), mating duration (i.e., the time between copulation initiation to termination) and mating success for each couple (i.e., gyne and drone detach from copula). Couples that did not start mating within 60 min were considered unsuccessful and the mating trial was aborted [56]. Unsuccessful males were granted a second and final opportunity to mate by placing the rejected drone in a new mating arena with a naive virgin gyne. Again, mating latency, duration, as well as mating success, were recorded. Immediately after the mating behaviour trial, all drones were dissected and the sperm traits (i.e., quantity and viability) of each individual were determined (see below), irrespective of mating success. Successfully mated gynes remained in their respective mating arenas with *ad libitum* 50% [w/w] sucrose-solution and pesticide-free corbicular honeybee pollen which was placed in the lid of an Eppendorf tube (SI Fig. 1C). To ensure successful migration of the sperm into the gyne's spermatheca, the gynes were transferred to an incubator at 28 °C and 60% RH in complete darkness for 24 h. Following Duvoisin et al. (1999), all gynes were dissected to assess sperm traits (see below).

2.4. Sperm assessments

Sperm quantity and sperm viability were assessed for a total of 110 drones (i.e., all drones from the mating trial irrespective of their mating success ($N = 49$) and all remaining drones surviving the exposure period and not used for the mating ($N = 61$)). Likewise, all gynes that were successfully mated were used for the sperm assessment ($N = 37$). Drones and gynes were briefly anesthetized using CO_2 before being pinned to a wax plate and dissected. Following established protocols [41,57], the entire drone genitalia (i.e., including the testis, accessory gland, *vesical seminalis,* and ejaculatory duct), as well as the gyne spermatheca (Fig. 1A–D), were carefully dissected and stored in $Kiev^+$ buffer solution individually. Sperm quantity (i.e., total concentration of sperm), sperm viability (i.e., proportion of sperm alive), and total living sperm (i.e., product of multiplying the determined sperm quantity by sperm viability) were assessed [33]. For those males that successfully mated, we added the sperm quantity determined in the spermatheca of the respective gyne partner to their post-mating sperm quantity to obtain an estimation of their total sperm quantity neglecting the possible surplus. Lastly, to measure the difference in sperm viability between mated couples, the sperm viability of the gynes was subtracted from the drone sperm viability.

2.5. Statistical analyses

Statistical analyses were performed using STATA16 [58], whereas all statistical figures were created using NCSS 20 [59]. All outcome variables were tested for normality by using the Shapiro-Wilk's test and homogeneity of variances with the Levene's test and subsequent statistical methods were chosen accordingly (see SI Tables 1 and 2). To assess potential relationships amongst explanatory variables (e.g., start and end mass, exposure, mating latency) and the dependent variables (i.e., sucrose consumption, body mass difference, sperm traits, mating latency, mating duration, and mating success) linear regression was applied using the function *regress*, where individual bees were considered independent units. Further, to determine differences between the control and neonicotinoid treatment, generalized logistic or linear (regression) models (GLMs) were fit using the functions *glm* or *ologit*. Again, individual bumblebees were considered independent units, and the treatment (neonicotinoid versus control) was included as the explanatory fixed term. Whenever necessary, co-variates (e.g., body mass, consumption, round, or drone sperm traits) were included in each model. For each model, a stepwise backward elimination approach was applied to determine the model of best fit. Best fit models were chosen by comparing every multi-level model to its single-level model counterpart using a likelihood ratio (LR) test and comparing different models with the Akaike information criterion (AIC) using the functions *lrtest* and *estat ic*, respectively. Whenever appropriate, the means ± standard error (SE) or medians ± 95% confidence intervals (CI) are given in the text as well as in the SI Table 3. Median differences and their 95% CI were calculated using the STATA16 package *somersd*. The function *cendif* calculates confidence intervals for Hodges–Lehmann median differences (or other percentile differences) between two groups.

Depending on the analysis of residuals for the consumption, body mass difference, mating behaviour, and sperm count and total living sperm variables, GLMs were modeled by adjusting for either Gaussian, Gamma, or Poisson distribution by adding the function *family*(*distribution*) (SI Table 2). Counter transforming the outcome variables if not normally distributed, we opted for the Gamma or Poisson distribution family that provided good fits (normality of the residuals). Logistic GLMs were applied to test for treatment differences for the binary outcome variable mating success [%] using the function *logit*, whereby the conditional distribution of the regression was assumed to be Bernoulli. As sperm viability is a score ranging from 0 to 100%, we opted to apply a proportioned ordered logistic model using the function *ologit*. Survival time was set using the function *stset* and the if option was used for censored individuals. Differences in survival amongst treatments were fitted using the *streg* function for multi-level survival models considering 'drone start mass' as a covariate [60] and data were plotted using Kaplan-Meier curves to visualize survival over time.

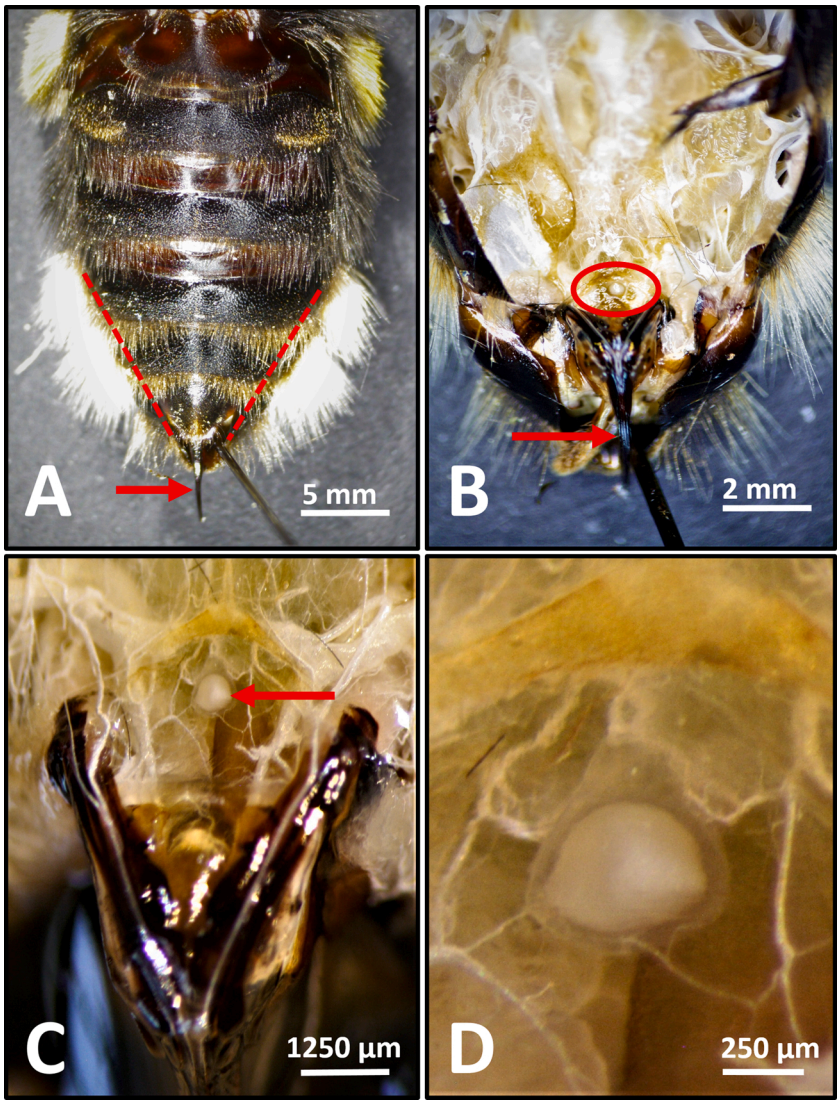

Fig. 1. Spermatheca dissection of a mated bumble bee gyne, *Bombus terrestris*. A) Ventral view of the abdomen prior to dissection. The red arrow points to the stinger of the gyne. Bilateral incisions were made using microscopy scissors at the caudal point of the abdomen starting from the beginning of the 6th to the end of the 4th tegeral segment (indicated by the red dashed lines) to reveal the inside of the abdomen. **B)** Ventral view of the abdominal cavity where the red arrow points towards the stinger and the red circle indicates the spermatheca. **C & D)** Enlarged view of the spermatheca.

For the vast majority of variables (e.g., sucrose consumption, body mass difference, mating duration, or drone sperm quantity), GLMs were modelled using either a Gaussian, Gamma or Poisson distribution, whereby 'treatment' was the single fixed term and either 'start mass [mg]' or 'sucrose consumption [g]' was included as a covariate. The logistic model for mating success [%] was based on a Bernouli distribution, where the fixed factor was 'treatment' and 'round' (i.e., first attempt or second attempt for the drone to mate with a gyne), as well as 'drone start mass' were included in the model as covariates. In addition, all drone sperm traits (i.e., sperm quantity, sperm viability, and total living sperm) were modelled using Gamma or proportioned ordered logistic distribution, where 'treatment' and 'success status' (i.e., if the drone had successfully mated or not) were included as the fixed terms and 'drone start mass' as a covariate. Drone success status was included because successful mating would evidently lower sperm counts in these individuals compared to non-successful individuals. All gyne sperm traits (i.e., sperm quantity, viability and total living sperm) were modelled using GLMs, where either a Gaussian or a proportioned ordered logistic distribution was applied. Again, 'treatment' was the fixed term, 'drone total living sperm [thousands]' and 'round' (i.e., first or second attempt of the drone to mate with a gyne) were included as a covariates. Further details to each model, including Shapiro-Wilk's results, applied distributions, covariates, fixed effects, as well as the STATA function used for each variable, can be found in the SI Table 2.

3. Results

3.1. Consumption, exposure, survival and body mass difference

Irrespective of the treatment group, body mass did not significantly correlate with pollen consumption ($F_{2,\ 100} = 2.04$, $R^2_{Adj.} = 0.01$, $t = -1.43$, $p = 0.16$; SI Table 1); whereas a significant positive correlation was observed between body mass and sucrose-solution consumption ($F_{2,\ 117} = 4.73$, $R^2_{Adj.} = 0.08$, $t = 2.21$, $p = 0.027$). Neonicotinoid exposure led to a significant reduction in pollen consumption (*glm*; $z = -2.68$, $p = 0.008$); resulting in thiamethoxam-exposed drones (0.09 ± 0.007) consuming ~25% less than controls (0.12 ± 0.008; SI Table 3; mean ± SE g). Likewise, thiamethoxam exposure revealed a significant negative effect on sucrose-solution consumption (*glm*; $z = -2.12$, $p = 0.034$; SI Table 2), with 3.55 ± 0.08 and 3.29 ± 0.09 g consumed by control and neonicotinoid drones, respectively (mean ± SE g; SI Table 3). This corresponds to a 7.3% reduction in sucrose-solution consumption by drones exposed to thiamethoxam. Neonicotinoid drones surviving the 12 day exposure period were on average exposed to 66.23 ± 1.82 ng of thiamethoxam (mean ± SE; SI Table 3). Neonicotinoid exposure revealed no significant effect on survival (*streg*; $\chi^2 = 0.62$, $z = 0.57$, $p = 0.57$; SI Table 2), where control and thiamethoxam-exposed drone cumulative survival post-exposure were 90.3± 3.8 and 87.1± 4.3%, respectively (mean ± SE %, SI Table 3). Lastly, irrespective of the

treatment group, body mass difference did not significantly correlate with pollen and sucrose-solution consumption or exposure ($F_{4, 94} = 5.20$, $R^2_{Adj.} = 0.15$ all t's < 0.98, all p's > 0.33; SI Table 1). However, a significant negative correlation was observed between start mass and body mass difference for both treatments ($F_{4, 94} = 5.20$, $R^2_{Adj.} = 0.15$, $t = -4.54$, $p < 0.001$; SI Table 1); whereby heavier drones were more likely to lose mass and in contrary lighter drones gained mass. Furthermore, no significant difference in body mass difference was observed between thiamethoxam-exposed and control drones (*glm*; $z = -0.37$, $p > 0.71$; SI Table 2), resulting in an average body mass difference of -22 mg ± 5.76 (mean ± S.E.; SI Table 3).

3.2. Mating latency, duration and success

Neither pollen and sucrose-solution consumption nor start mass revealed a significant correlation with mating latency ($F_{4, 31} = 0.17$, $R^2_{Adj.} = 0.044$, all t's< 1.7, all p's> 0.1, SI Table 1). However, neonicotinoid exposure led to a significantly reduced mating latency compared to controls (*glm*; $z = -4.69$, $p < 0.001$; Fig. 2A, SI Table 2); where mating latency was 7.18 ± 0.65 and 12 ±0.74 min for neonicotinoids and controls, respectively (SI Table 3; mean ± SE min). Subsequently, thiamethoxam-exposed drones that successfully mated required 40% less time to start copulating with their gyne partner. Further, while pollen consumption and start mass did not affect mating duration ($F_{3, 31} = 2.1$, $R^2_{Adj.} = 0.11$, $t < 1.83$, $p > 0.08$, SI Table 1), sucrose-solution consumption was significantly positively correlated with mating duration ($t = 2.58$, $p < 0.02$). Nevertheless, mating duration was not significantly affected by thiamethoxam exposure (*glm*; $z = 0.84$, $p = 0.40$; Fig. 2B, SI Table 2), where the average mating across both treatments lasted 26.59 ± 1.09 min (mean ±SE, SI Table 3).

Neither consumption nor start mass revealed a significant effect on mating success ($F_{3, 31} = 0.16$, $R^2_{Adj.} = 0.04$, all t's < 1.7, all p's > 0.10, SI Table 1). In contrast, neonicotinoid exposure significantly reduced drone mating success (*logit*; $z = -2.22$, $p = 0.026$; SI Table 2). However, the level of exposure revealed no significant effect on mating success ($F_{1, 31} = 1.69$, $R^2_{Adj.} = 0.04$, $t = -1.57$, $p = 0.122$, SI Table 1). During the first round of mating, 62 ± 0.1% of the thiamethoxam-exposed drones successfully mated whereas the control success rate was 74 ± 0.09% (Table 1, mean ± SE). The reduced mating success was further evident after the second round, where only 10 ± 0.10% of the non-successful neonicotinoid drones mated, in sharp contrast to the 83 ± 0.16% observed in the controls (Table 1, mean ± SE). Overall, control and neonicotinoid mating success were 96 ± 0.04% and 65 ± 0.09%, respectively (Table 1; mean ± SE); resulting in a reduction of 32% due to neonicotinoid exposure.

Table 1
Effects of neonicotinoid insecticide (thiamethoxam) exposure on mating success of male (drone) bumblebees, *Bombus terrestris*. A subset of drones from the control ($N = 23$) and neonicotinoid treatment ($N = 26$) were randomly selected for a mating behaviour trial. If drones did not start mating after 60 min with the virgin gyne the mating was considered unsuccessful (Round 1); the mating trial was then aborted and the pair were separated. The unsuccessful males were granted a second and final opportunity to mate (Round 2). Thiamethoxam exposure significantly reduced drone mating success ($p = 0.026$); revealing a 95.65 and 65.38% for controls and neonicotinoid insecticide drones, respectively. This corresponds to a reduction in mating success of approximately 30%.

Round	Treatment	Sample size	Mated	Non-mated	Mating success [%]
1	Control	23	17	6	73.91
	Neonicotinoid	26	16	10	61.53
2	Control	6	5	1	83.33
	Neonicotinoid	10	1	9	10.00
Overall	Control	23	22	1	95.65
	Neonicotinoid	26	17	9	65.38

3.3. Sperm assessments

None of the measured explanatory variables (i.e., pollen/sucrose-solution consumption, start mass or body mass difference) revealed a significant effect on any of the tested sperm traits for either drones or within the gynes (all p's > 0.19, SI Table 1). However, a significantly negative correlation was observed between control drone sperm quantity and viability ($F_{1, 28} = 5.22$, $R^2_{Adj.} = 0.127$, $t = -2.28$, $p = 0.03$), reflecting lower sperm viability as sperm counts increased.

3.3.1. Drones

A significant difference was observed between thiamethoxam-exposed and control drone sperm quantity (*glm*; $z = -2.16$, $p = 0.031$; Fig. 3A, SI Table 2), with 271 ± 181–363 and 325 ± 187–462 thousand sperm, respectively (median ± 95% CI, SI Table 3). Therefore, thiamethoxam-exposed drones exhibited a reduction in sperm quantity by ~17%. Likewise, drone sperm viability was significantly different between treatments (*ologit*; $z = -5.11$, $p < 0.001$; Fig. 3B, SI Table 2). Thiamethoxam-exposed drones possessed lower sperm viability (76 ± 72.6–79.8%) than controls (85 ± 82.5–87.9%), reflecting a reduction in sperm viability of ~10% (median ± 95% CI, SI Table 3). Furthermore, total living sperm quantity was significantly different between the two treatment groups (*glm*; $z = -3.75$, $p < 0.001$; Fig. 3C, SI Table 2), with thiamethoxam-exposed drones possessing ~23% less living sperm than controls. Living sperm quantity was 213 ± 140–286 and 276 ± 164–387 thousand in the neonicotinoids and controls, respectively (median ± 95% CI, SI Table 3). Irrespective of the treatment group, the sperm

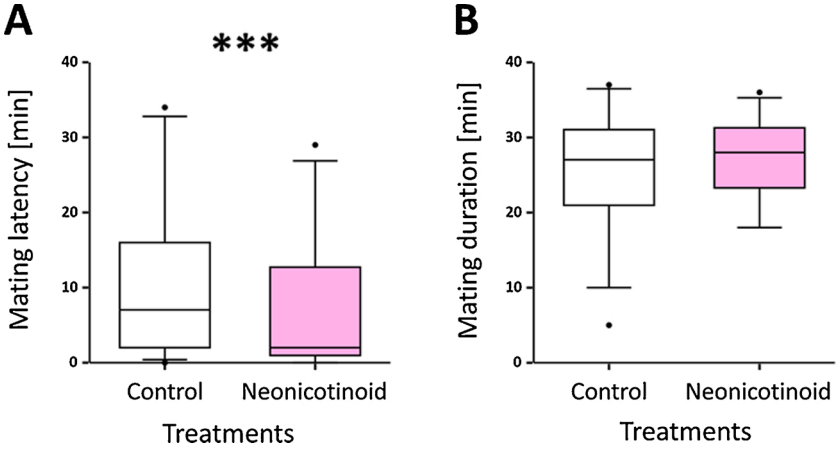

Fig. 2. Effects of neonicotinoid insecticide (thiamethoxam) exposure on mating behaviour of male (drone) bumblebees, *Bombus terrestris*. (A) Neonicotinoid exposure led to a significantly reduced mating latency compared to controls ($p < 0.001$); where thiamethoxam-exposed drones required roughly 40% less time to successfully copulate with the gyne. **(B)** In contrast, neonicotinoid exposure did not significantly affect mating duration ($p = 0.40$); where the average mating lasted 26.6 min for both treatments. Boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots). A significant difference between treatment groups is indicated by *** ($p < 0.001$).

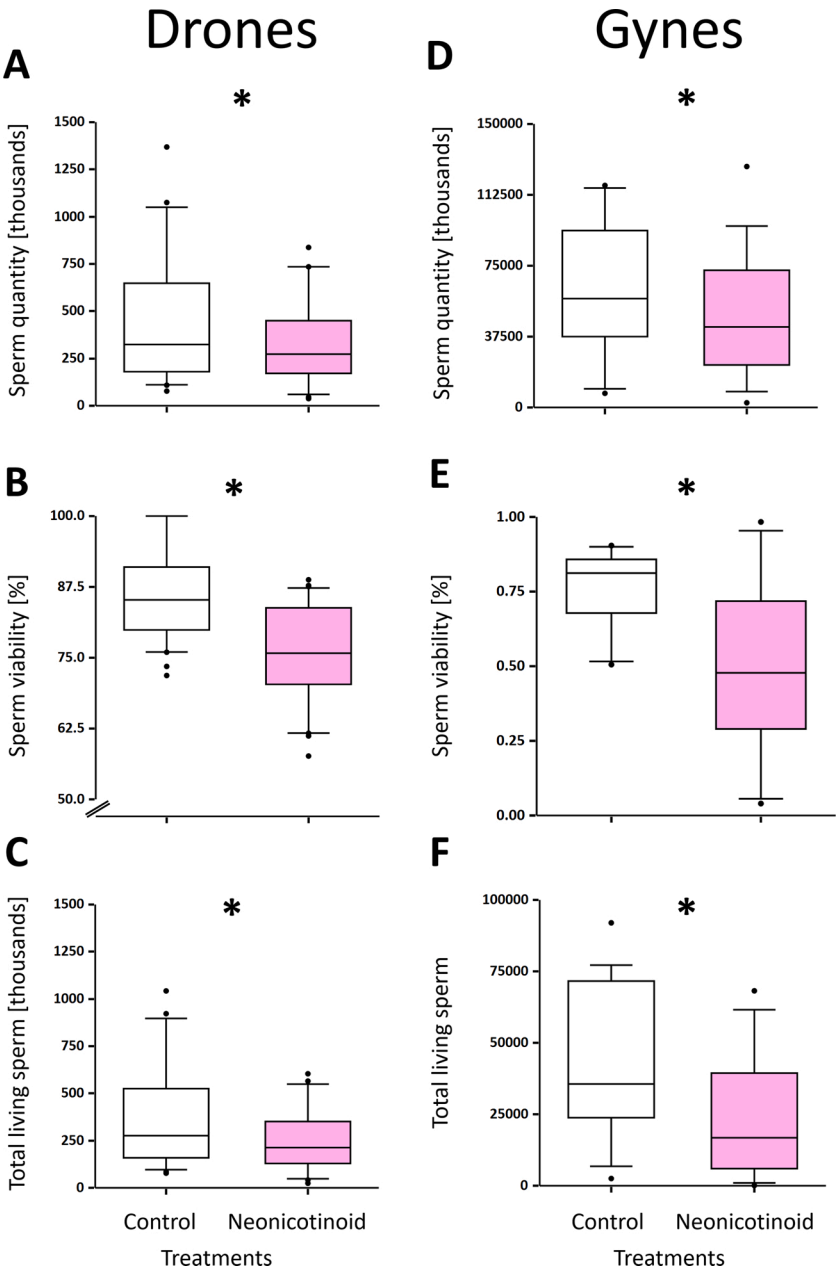

Fig. 3. Sperm assessment in male (drone) and in spermathecae of female (gyne) bumblebees, *Bombus terrestris*, exposed to the neonicotinoid insecticide thiamethoxam: (A&D) Comparison of sperm quantity in both drones and gynes revealed a significant difference ($p < 0.05$) with lower values for thiamethoxam-exposed individuals. **(B&E)** Percentage of viable sperm in both drones and gynes showed significant differences ($p < 0.05$) with lower values for thiamethoxam-exposed individuals **(C&F)** A significant difference in total living sperm quantity was observed for both drones and gynes ($p < 0.01$) with lower values for thiamethoxam-exposed individuals. Boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots). A significant difference between treatment groups is indicated by * ($p < 0.05$).

quantity and total living sperm significantly differ between successful and non-successful drones (*glm's*; all z's < -2.72, all p's < 0.002), whereas this was not the case for sperm viability (*glm*; $z = 0.44$, $p = 0.659$; SI Table 2).

3.3.2. *Gynes*

All sperm traits from gynes that had successfully mated with a thiamethoxam-exposed drone were significantly reduced in comparison to their control counterparts (all p's < 0.046, SI Table 2). Control-mated gyne sperm quantity (57.5 ± 36–79) was significantly higher when compared to the neonicotinoid-mated group (42.5 ± 22–63) (*glm*; $z = -2.0$, p ` $= 0.046$; Fig. 3D, SI Table 2; median ± 95% CI, SI Table 3); resulting in 26% fewer sperm for neonicotinoid-mated compared to control-mated gynes. Furthermore, control-mated gyne sperm viability (81.3 ± 74–89%) significantly differed from the neonicotinoid-mated gynes (47.8 ± 30–66%) (*ologit*; $z = -2.03$, $p = 0.042$; Fig. 3E, SI Table 2); resulting in a reduction of 34% (median ± 95% CI; SI Table 3). Furthermore, sperm viability in the control-mated gynes was 9.02 ± 43.8% lower when compared to their respective drone partner. In sharp contrast, the difference in sperm viability between neonicotinoid-mated gynes and their drones was −25.45 ± 7.71%; corresponding to a 2.82 fold reduction in sperm viability. Subsequently, the sperm viability difference between mated couples was significantly higher in the neonicotinoid treatment (*glm*; $z = -2.12$, $p = 0.034$). Lastly, control-mated gyne total living sperm quantity (47.8 ± 0.05) significantly differed from the neonicotinoid-mated gyne treatment group (23.9 ± 0.04) (*glm*; $z = -2.08$, $p = 0.038$; Fig. 3F, SI Table 2), with neonicotinoid-mated gynes possessing roughly 50% less living sperm (median ± SE, SI Table 3).

4. Discussion

The data demonstrate for the first time that neonicotinoid insecticides can impair male mating behaviour and reproductive success in bees. The field-realistic exposure of drones to thiamethoxam not only led to 32% fewer copulations, but also reduced their total living sperm

quantity by 23%. This evident effect on sperm quality became further prominent in the females inseminated by exposed males, as they revealed a 50% reduction in total living sperm quantity in the spermatheca compared to their respective controls. Given the apparent key role of sex and the indispensable role of functional males, the data are of particular concern in light of ubiquitous neonicotinoid contaminations and thus may constitute a possible mechanistic explanation for recent bumblebee population declines [61].

Chronic thiamethoxam exposure significantly reduced pollen and sucrose-solution consumption confirming previous findings [62,63]. This may be due to neurotoxicity reducing ability or willingness to feed [28,64] and/or avoidance of contaminated food [65]. Such nutrient deprivation can impair physiological development and detoxification abilities [66,67] leading to synergistic adverse effects when combined with thiamethoxam [68]. However, the consumption variables neither significantly impacted body mass difference nor mating behaviour or sperm traits. Therefore, nutriet deprivation appears an unlikely factor explaining later findings. Average daily exposure to 5.52 ng g^{-1} of thiamethoxam revealed no significant effect on bumblebee survival, confirming [33]. However, given that drones can live for several weeks [69], more data are required over longer durations and under field conditions. Nevertheless, our data support that neonicotinoids may not necessarily affect survival, yet can substantially impair essential parameters of bumblebee fitness (e.g., colony initiation, oviposition or production of sexuals) [29,46]. Such trade-offs between survival and fitness parameters are well-known [70] and may be due to costly detoxification at the expense of other traits [71].

The data demonstrate that neonicotinoid exposure can impair male bee mating behaviour. While our experiment would have benefited from using an experimental mate-choice design (i.e., females are offered both a control and thiamethoxam-exposed male simultaneously), our design nevertheless provides a key advantage because it focuses on the female's assessment of whether she considers the male as adequate. As rape under the given conditions is highly unlikely, due to the female's ability to sting and kill the male [72], successful copulation implies that the male was considered a worthy partner due to female choice. As neonicotinoid insecticides act as agonists of nicotinic acetylcholine receptors and thereby increasing neuronal activity [23], the observed reduced mating latency in exposed males may be linked to hyperactivity [24]. Further, while only being speculative, the reduced latency may also be linked to the drones self-assessment abilities as shown in honeybee queens [73]. Males exposed to thiamethoxam may have been aware of their intoxication and likely reduced abilities to mate, and thus were extremely eager to seize the opportunity to mate with a female. In contrast, thiamethoxam exposure did not significantly affect the duration of a copulation, wherein the overall mean mating duration (~27 min) lies within the range of previous studies [56,74]. Therefore, our data underline that mating duration is likely constrained and essential for copulation success [72].

Bumblebee gynes are selective in their mating behaviour and are known to reject a high proportion of males under both laboratory as well as field conditions [41,75]. Despite the laboratory conditions not enabling males to display precopulatory behaviours (i.e., scent-marking, guarding or patrolling [39]), the observed mating success of control males was overall very high (96%). This suggests that the control males were of high quality, even though previous studies showed optimal male and gyne mating age being between 7–12 days and 6–7 days, respectively [69,76]. Most importantly, the age of the males and gynes in our experiment did not differ between treatments and controls. Therefore, the striking differences in mating success despite the relatively low sample size indicate a strong treatment effect. Indeed, the thiamethoxam-exposed drones revealed 32% fewer successful matings when compared to the controls. This may be due to disruptions of chemical communication systems and/or visual or sensory behavioural cues (e.g., antennation [77]). Cuticular hydrocarbons (CHCs) can play a key role in insect mating recognition [78], and similar to previous observations in cockroaches and beetles [79,80], thiamethoxam may disrupt the CHC profile of males making them less attractive. Likewise, it remains to be tested if neonicotinoids can impair the volatile secretions from the cephalic labial glands of bumblebee males, which are essential in producing sex pheromones [81]. Such neurotoxic effects have previously been described for moths exposed to sublethal concentrations of the neonicotinoid thiacloprid [36]. Also, it remains to be tested if neonicotinoid exposure may hinder the male's ability to detect female chemical cues, including specific sex pheromones [35]. However, under the given laboratory conditions this likely would not have played a key role. Furthermore, displaying altered or inappropriate mating behaviour may also significantly reduce the chances of successfully mating, as previously observed in spiders [37] as well as bed bugs [82] exposed to sublethal neonicotinoid dosages. The reduced latency observed in thiamethoxam-exposed bumblebee males may indeed be disadvantageous as females may have perceived this as being too pushy (i.e., anthropologically speaking: 'non-gentleman-like'); thereby reducing male mating success. Lastly, neonicotinoid-exposed males may have a reduced ability to learn from their initial mistakes made during their first sexual experiences. The drastic increase in control mating success after initial failure compared to exposed males may be depicted as a case of improved learning after a negative feedback [83]. Similar negative effects of neonicotinoids on the learning ability of bumblebees has been reported [84,85] and may contribute to the low second round mating success of thiamethoxam-exposed males. Future studies should focus on understanding the mechanisms underlying the observed negative effects of neonicotinoids on male mating success, as well as test how neonicotinoids may affect female mate choice and behaviour. Indeed, increasing evidence suggests that that neonicotinoid exposure can compromise reproductive physiology and mating behaviour in females of various bee species [31,46,86].

The data confirm that thiamethoxam exposure can elicit sublethal effects on sperm traits [32,33], revealed by the reduction in drone sperm quantity, viability and total living sperm quantity by 17%, 10% and 23% compared to controls, respectively. The reduced sperm quantity suggests that thiamethoxam may hinder the migration of sperm from the testis to the seminal vesicles in bumblebees, as spermatogenesis and spermiogenesis are both completed upon emergence in bees [87]. Past studies on honeybee, *A. mellifera*, drones revealed no reduction in sperm quantity after neonicotinoid exposure during larval development or adulthood [88,89]. This apparent difference in susceptibility may be attributed to species-specific differences in reproductive physiology, behaviour, and life-history. In sharp contrast to honeybees, males of the genus *Bombus*, similar to other bee genera, are polygynous [90]; subsequently, any reduction in sperm quantity constitutes a constraint on fitness as their insemination capacity is reduced. Considering *B. terrestris* gynes store ~40′000 spermatozoa in their spermatheca [74], the observed control male sperm quantity would be sufficient to inseminate at least eight gynes, confirming previous observations [74]. In contrary, sperm quantity for the thiamethoxam-exposed males would infer that a maximum of six gynes could sufficiently be inseminated; reflecting a reduction in male fitness by 25%. Besides producing sufficient spermatozoa, it is crucial that the transferred sperm is of high quality. In line with previous studies [33,89], thiamethoxam-exposure led to a reduction in sperm viability by 10%. This effect may be due to impaired sperm mitochondria and seminal fluid protein, or increased oxidative stress, as previously shown for honeybees [88,91]. Taken together, the reduced sperm quantity and viability led to 23% fewer total living sperm. While only a small fraction of transferred sperm is stored in the female spermatheca, any decrease in living sperm quantity could have negative consequences on fertilization success and the production of female offspring.

Indeed, females inseminated by thiamethoxam-exposed males revealed 23% fewer and 34% less viable sperm in the spermatheca when compared to the control-inseminated females. Therefore, the sperm traits were even further compromised after copulation and migration to

the spermatheca. The reduced sperm quantity may not necessarily reflect an evident constraint [57], as females of highly eusocial but also solitary bee species store excess spermatozoa in their spermatheca [92,93], and only a small fraction of the sperm is required to successfully complete a colony cycle [74]. While it remains speculative as to why the sperm viability in the spermatheca of neonicotinoid-inseminated queens was a 3.75 fold lower than the controls; this effect may be attributed to neonicotinoids impairing the motility of viable sperm thereby hindering the sperm to actively migrate [88] and/or decreasing protein content in semen required to ensure long-term storage in the spermatheca [57,72]. Furthermore, as long-term sperm storage is indeed costly [94], females may limit the nourishment of sperm in the spermatheca to maintain the immune system at the cost of letting sperm die [95]. Indeed, our data provide further support for this trade-off as both treatments revealed significantly lower sperm viability when compared to their male counterparts. Ultimately, the reduced female sperm quantity and viability resulted in a 50% reduction in total living sperm quantity in the thiamethoxam-inseminated female spermathecae. Such drastically reduced viable sperm availability may increase the likelihood of producing non-intentional males. This would reveal particularly disadvantageous during the critical stage of colony initiation, when gynes depend on the first generation of workers to assist in colony related tasks (i.e., brood care and foraging) and growth. Impaired colony development will lead to less sexuals being produced [29], especially gynes, which constitutes an additional long-term fitness constraint on the males and ultimately the entire populations.

In conclusion, the reduced mating success in combination with reduced sperm traits reflects a drastic scenario for bumblebee fitness, irrespective of the underlying mechanism. Given our laboratory findings can be extrapolated to the field and hold true across various insect taxa, which appears likely due to the non-specific mode of action and widespread neonicotinoid pollution globally [25,96], this may help understand recent insect population declines; especially for those species where unfertilized eggs do not hatch [97,98]. Therefore, to fully understand the potential threat of xenobiotic substances on ecosystems, we urge risk assessments to determine the ecological effects on fitness of individuals, colonies and entire populations [99,100].

Author contribution

L.S and V.S. designed the experiment. L.S., A.M., D.C., I.K., and V.S. collected the data; L.S., A.V.O., F.W., and P.N. provided materials and reagents; L.S., A.V.O., and V.S. designed the statistical analysis; L.S., P. N., and V.S. analysed the data and wrote the manuscript. All authors discussed the results and contributed to editing and approving the manuscript.

Data accessibility

The complete raw data can be found at the Dryad repository. See: https://doi.org/10.5061/dryad.3nvx0k6dsh

Funding

Support was provided by the Swiss Federal Office of the Environment (FOEN) to L.S., A.M., and P.N., (16.0091.PJ/R102-1664), by Agroscope to L.S. and P.N., and by the Vinetum Foundation to P.N. and V.S. We sincerely thank the University of Bern for providing Open Access funding.

Conflict of Interest

The authors declare no conflict of interest.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

We would like to thank Maria a Marca and Christoph Moor from the Swiss Federal Office of the Environment (FOEN) for their great support and for the engaged fertile discussions; Lukas Jeker from the Swiss Bee Research Centre, Agroscope, for superb support with preparing insecticide solutions and for sharing his know-how.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.12.003.

References

[1] J.M. Smith, J. Maynard-Smith, The Evolution of Sex, Cambridge University Press, Cambridge, 1978.
[2] B.S. Walsh, S.R. Parratt, A.A. Hoffmann, D. Atkinson, R.R. Snook, A. Bretman, T. A.R. Price, The impact of climate change on fertility, Trends Ecol. Evol. 34 (2019) 249–259, https://doi.org/10.1016/j.tree.2018.12.002.
[3] T.A. Wehr, Photoperiodism in humans and other primates: evidence and implications, J. Biol. Rhythms 16 (2001) 348–364, https://doi.org/10.1177/074873001129002060.
[4] R.M. Sharpe, S. Franks, Environment, lifestyle and infertility - an intergenerational issue, Nat. Med. 8 (2002), https://doi.org/10.1038/nm-fertilityS33.
[5] M.C. Metz, D.J. Emlen, D.R. Stahler, D.R. MacNulty, D.W. Smith, M. Hebblewhite, Predation shapes the evolutionary traits of cervid weapons, Nat. Ecol. Evol. 2 (2018) 1619–1625.
[6] S.H. Swan, S. Colino, Count Down, how our modern world is threatening sperm counts, altering male and female reproductive development, and imperiling the future of the human race, Scribner (2021).
[7] P.J. Landrigan, R. Fuller, N.J.R. Acosta, O. Adeyi, R. Arnold, N.(Nil) Basu, A. B. Baldé, R. Bertollini, S. Bose-O'Reilly, J.I. Boufford, P.N. Breysse, T. Chiles, C. Mahidol, A.M. Coll-Seck, M.L. Cropper, J. Fobil, V. Fuster, M. Greenstone, A. Haines, D. Hanrahan, D. Hunter, M. Khare, A. Krupnick, B. Lanphear, B. Lohani, K. Martin, K.V. Mathiasen, M.A. McTeer, C.J.L. Murray, J. D. Ndahimananjara, F. Perera, J. Potočnik, A.S. Preker, J. Ramesh, J. Rockström, C. Salinas, L.D. Samson, K. Sandilya, P.D. Sly, K.R. Smith, A. Steiner, R.B. Stewart, W.A. Suk, O.C.P. van Schayck, G.N. Yadama, K. Yumkella, M. Zhong, The Lancet Commission on pollution and health, Lancet 391 (2018) 462–512, https://doi.org/10.1016/S0140-6736(17)32345-0.
[8] H.C.J. Godfray, A.E.A. Stephens, P.D. Jepson, S. Jobling, A.C. Johnson, P. Matthiessen, J.P. Sumpter, C.R. Tyler, A.R. McLean, A restatement of the natural science evidence base on the effects of endocrine disrupting chemicals on wildlife, Proc. R. Soc. B Biol. Sci. 286 (2019), https://doi.org/10.1098/rspb.2018.2416.
[9] R. De Celis, N. Pedrón-Nuevo, A. Feria-Velasco, Toxicology of male reproduction in animals and humans, Arch. Androl. 37 (1996) 201–218, https://doi.org/10.3109/01485019608988523.
[10] H. Merzenich, H. Zeeb, M. Blettner, Decreasing sperm quality: A global problem? BMC Public Health 10 (2010) https://doi.org/10.1186/1471-2458-10-24.
[11] D.M. Fry, Reproductive effects in birds exposed to pesticides and industrial chemicals, Environ. Health Perspect. 103 (1995) 165–171, https://doi.org/10.2307/3432528.
[12] C. Castellini, M. Totaro, A. Parisi, S. D'Andrea, L. Lucente, G. Cordeschi, S. Francavilla, F. Francavilla, A. Barbonetti, Bisphenol A and male fertility: myths and realities, Front. Endocrinol. 11 (2020) 1–10, https://doi.org/10.3389/fendo.2020.00353.
[13] I.C. Oskam, E. Ropstad, E. Dahl, E. Lie, A.E. Derocher, Ø. Wiig, S. Larsen, R. Wiger, J.U. Skaare, Organochlorines affect the major androgenic hormone, testosterone, in male polar bears (*Ursus maritimus*) at Svalbard, J. Toxicol. Environ. Heal. - Part A. 66 (2003) 2119–2139, https://doi.org/10.1080/15287390390211342.
[14] T.B. Hayes, M. Hansen, From silent spring to silent night: agrochemicals and the anthropocene, Elem. Sci. Anth. 5 (2017) 1–24, https://doi.org/10.1525/elementa.246.
[15] M.I. Yousef, M.H. Salem, S. Helmi, M.A. Seehy, K. Bertheussen, Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits, J. Environ. Sci. Heal. Part B Pestic. Food Contam. Agric. Wastes. 30 (1995) 513–534.
[16] P. Frederick, N. Jayasena, Altered pairing behaviour and reproductive success in white ibises exposed to environmentally relevant concentrations of methylmercury, Proc. R. Soc. B Biol. Sci. 278 (2011) 1851–1857, https://doi.org/10.1098/rspb.2010.2189.
[17] T.M. Edwards, B.C. Moore, L.J. Guillette, N. Olea, J. McLachlan, D. Page, Reproductive dysgenesis in wildlife: a comparative view, Int. J. Androl. 29 (2006) 109–121, https://doi.org/10.1111/j.1365-2605.2005.00631.x.

[18] A. Sharma, V. Kumar, B. Shahzad, M. Tanveer, G.P.S. Sidhu, N. Handa, S.K. Kohli, P. Yadav, A.S. Bali, R.D. Parihar, O.I. Dar, K. Singh, S. Jasrotia, P. Bakshi, M. Ramakrishnan, S. Kumar, R. Bhardwaj, A.K. Thukral, Worldwide pesticide usage and its impacts on ecosystem, SN Appl. Sci. 1 (2019) 1–16, https://doi.org/10.1007/s42452-019-1485-1.

[19] D.L. Wagner, Insect declines in the anthropocene, Annu. Rev. Entomol. 65 (2020) 457–480, https://doi.org/10.1146/annurev-ento-011019-025151.

[20] P. Jeschke, R. Nauen, M. Schindler, A.E. X, Overview of the status and global strategy for neonicotinoids, Agric. Food Chem. 59 (2011) 2897–2908.

[21] F. Sánchez-Bayo, K.A.G. Wyckhuys, Further evidence for a global decline of the entomofauna, Austral Entomol. 60 (2021) 9–26, https://doi.org/10.1111/aen.12509.

[22] B.A. Woodcock, N.J.B. Isaac, J.M. Bullock, D.B. Roy, D.G. Garthwaite, A. Crowe, R.F. Pywell, Impacts of neonicotinoid use on long-term population changes in wild bees in England, Nat. Commun. 7 (2016) 12459, https://doi.org/10.1038/ncomms12459.

[23] K. Matsuda, M. Ihara, D.B. Sattelle, Neonicotinoid insecticides: molecular targets, resistance, and toxicity, Annu. Rev. Pharmacol. Toxicol. 60 (2020) 241–255, https://doi.org/10.1146/annurev-pharmtox-010818-021747.

[24] S. Tosi, C. Costa, U. Vesco, G. Quaglia, G. Guido, A 3-year survey of Italian honey bee-collected pollen reveals widespread contamination by agricultural pesticides, Sci. Total Environ. 615 (2018) 208–218, https://doi.org/10.1016/j.scitotenv.2017.09.226.

[25] M. Mörtl, Á. Vehovszky, S. Klátyik, E. Takács, J. Győri, A. Székács, Neonicotinoids: spreading, translocation and aquatic toxicity, Int. J. Environ. Res. Public Health 17 (2020), https://doi.org/10.3390/ijerph17062006.

[26] F. Sgolastra, P. Medrzycki, L. Bortolotti, S. Maini, C. Porrini, Bees and pesticide regulation: lessons from the neonicotinoid experience, Biol. Conserv. 241 (2020), 108356, https://doi.org/10.1016/j.biocon.2019.108356.

[27] S. Tosi, G. Burgio, J.C. Nieh, A common neonicotinoid pesticide, thiamethoxam, impairs honey bee flight ability, Sci. Rep. 7 (2017) 1201, https://doi.org/10.1038/s41598-017-01361-8.

[28] S.M. Williamson, S.J. Willis, G.A. Wright, Exposure to neonicotinoids influences the motor function of adult worker honeybees, Ecotoxicology 23 (2014) 1409–1418, https://doi.org/10.1007/s10646-014-1283-x.

[29] P.R. Whitehorn, S.O. 'Connor, F.L. Wackers, D. Goulson, Neonicotinoid pesticide reduces bumble bee colony growth and queen production, Science 336 (6079) (2012) 351–352, https://doi.org/10.1126/science.1215025.

[30] C. Sandrock, L.G. Tanadini, J.S. Pettis, J.C. Biesmeijer, S.G. Potts, P. Neumann, Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success, Agric. For. Entomol. 16 (2014) 119–128, https://doi.org/10.1111/afe.12041.

[31] F. Sgolastra, X. Arnan, R. Cabbri, G. Isani, P. Medrzycki, D. Teper, J. Bosch, S. Mediche, Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee, Proc. R. Soc. B. 285 (2018) 1–8.

[32] V. Strobl, M. Albrecht, L. Villamar-Bouza, S. Tosi, P. Neumann, L. Straub, The neonicotinoid thiamethoxam impairs male fertility in solitary bees, *Osmia cornuta*, Environ. Pollut. 284 (2021), 117106, https://doi.org/10.1016/j.envpol.2021.117106.

[33] A. Minnameyer, V. Strobl, S. Bruckner, D.W. Camenzind, A. Van Oystaeyen, F. Wäckers, G.R. Williams, O. Yañez, P. Neumann, L. Straub, Eusocial insect declines: insecticide impairs sperm and feeding glands in bumblebees, Sci. Total Environ. 785 (2021), 146955, https://doi.org/10.1016/j.scitotenv.2021.146955.

[34] L. Tappert, T. Pokorny, J. Hofferberth, J. Ruther, Sublethal doses of imidacloprid disrupt sexual communication and host finding in a parasitoid wasp, Sci. Rep. 7 (2017) 1–9, https://doi.org/10.1038/srep42756.

[35] A.N. Kremer, B.H. King, A neonicotinoid affects the mating behavior of *Spalangia endius* (Hymenoptera: pteromalidae), a biological control agent of filth flies, Environ. Entomol. 48 (2019) 489–495, https://doi.org/10.1093/ee/nvz015.

[36] M.A. Navarro-Roldán, C. Gemeno, Sublethal effects of neonicotinoid insecticide on calling behavior and pheromone production of Tortricid moths, J. Chem. Ecol. 43 (2017) 881–890, https://doi.org/10.1007/s10886-017-0883-3.

[37] S. Korenko, J. Sýkora, M. Řezáč, P. Heneberg, Neonicotinoids suppress contact chemoreception in a common farmland spider, Sci. Rep. 10 (2020) 1–8, https://doi.org/10.1038/s41598-020-63955-z.

[38] D. Goulson, P.A. Marchand, S. Geisen, Call to restrict neonicotinoids, Science 360 (6392) (2018) 973, https://doi.org/10.1126/science.aau0432.

[39] D. Goulson, Bumblebees. Behaviour, Ecology, and Conservation, 2nd ed., Oxford Univ. Press, Oxford, 2003.

[40] M.J.F. Brown, B. Baer, R. Schmid-Hempel, P. Schmid-Hempel, Dynamics of multiple-mating in the bumble bee *Bombus hypnorum*, Insectes Soc. 49 (2002) 315–319, https://doi.org/10.1007/PL00012654.

[41] N. Duvoisin, B. Baer, P. Schmid-Hempel, Sperm transfer and male competition in a bumblebee, Anim. Behav. (1999) 743–749, 58.

[42] F. Ruttner, G. Koeniger, Die Füllung der Spermatheka der Bienenkönigin, Z. Vergl. Physiol. 72 (1971) 411–422.

[43] E. Bresslau, Der Samenblasengang der Bienenkönigin, Zool. Anz. 29 (1905) 299–325.

[44] F. Ruttner, The mating of the honeybee, Bee World 37 (1956) 3–15, https://doi.org/10.1080/0005772X.1956.11094913.

[45] A.N. Arce, T.I. David, E.L. Randall, A. Ramos Rodrigues, T.J. Colgan, Y. Wurm, R. J. Gill, Impact of controlled neonicotinoid exposure on bumblebees in a realistic field setting, J. Appl. Ecol. 54 (2017) 1199–1208, https://doi.org/10.1111/1365-2664.12792.

[46] J. Wu-Smart, M. Spivak, Effects of neonicotinoid imidacloprid exposure on bumble bee (Hymenoptera: apidae) queen survival and nest initiation, Environ. Entomol. 47 (2018) 55–62, https://doi.org/10.1093/ee/nvx175.

[47] D.V. Alford, Bumblebees, Davis-Poynter, Minnesota, 1975. https://books.google.ch/books?id=KzcgAQAAMAAJ.

[48] C. Botias, A. David, J. Horwood, A. Abdul-Sada, E. Nicholls, E. Hill, D. Goulson, Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees, Environ. Sci. Technol. 49 (2015) 12731–12740, https://doi.org/10.1021/acs.est.5b03459.

[49] E. Pilling, P. Campbell, M. Coulson, N. Ruddle, I. Tornier, A four-year field program investigating long-term effects of repeated exposure of honey bee colonies to flowering crops treated with thiamethoxam, PLoS One 8 (2013) e77193, https://doi.org/10.1371/journal.pone.0077193.

[50] K.A. Stoner, B.D. Eitzer, Movement of soil-applied imidacloprid and thiamethoxam into nectar and pollen of squash (*Cucurbita pepo*), PLoS One 7 (2012) 1–5, https://doi.org/10.1371/journal.pone.0039114.

[51] T.J. Wood, I. Kaplan, Y. Zhang, Z. Szendrei, Honeybee dietary neonicotinoid exposure is associated with pollen collection from agricultural weeds, Proc. R. Soc. B Biol. Sci. 286 (2019), https://doi.org/10.1098/rspb.2019.0989.

[52] I. Hrynko, B. Łozowicka, P. Kaczyński, Development of precise micro analytical tool to identify potential insecticide hazards to bees in guttation fluid using LC–ESI–MS/MS, Chemosphere. 263 (2021), https://doi.org/10.1016/j.chemosphere.2020.128143.

[53] M. Calvo-Agudo, J. González-Cabrera, Y. Picó, P. Calatayud-Vernich, A. Urbaneja, M. Dicke, A. Tena, Neonicotinoids in excretion product of phloem-feeding insects kill beneficial insects, Proc. Natl. Acad. Sci. U. S. A. 116 (2019) 16817–16822, https://doi.org/10.1073/pnas.1904298116.

[54] A.D. Vaudo, D. Stabler, H.M. Patch, J.F. Tooker, C.M. Grozinger, G.A. Wright, Bumble bees regulate their intake of essential protein and lipid pollen macronutrients, J. Exp. Biol. 220 (2017) 507, https://doi.org/10.1242/jeb.155911.

[55] J. Belsky, N.K. Joshi, Effects of fungicide and herbicide chemical exposure on *Apis* and non-*Apis* bees in agricultural landscape, Front. Environ. Sci. 8 (2020) 1–10, https://doi.org/10.3389/fenvs.2020.00081.

[56] M.R. Amin, L.F. Bussière, D. Goulson, Effects of male age and size on mating success in the bumblebee *Bombus terrestris*, J. Insect Behav. 25 (2012) 362–374, https://doi.org/10.1007/s10905-011-9306-4.

[57] B. Baer, P. Schmid-Hempel, The artificial insemination of bumblebee queens, Insectes Soc. 47 (2000) 183–187, https://doi.org/10.1007/PL00001699.

[58] StataCorp, STATA 16, 2019. Stata Statistical Software: Release 16.

[59] J. Hintze, NCSS 2020, 2020. ncss.com/software/ncss.

[60] G. Leckie, Centre for multilevel modeling, LEMMA VLE, Cent. Multilevel Model. (2010).

[61] D. Goulson, G.C. Lye, B. Darvill, Decline and conservation of bumble bees, Annu. Rev. Entomol. 53 (2008) 191–208, https://doi.org/10.1146/annurev.ento.53.103106.093454.

[62] R.J. Gill, O. Ramos-Rodriguez, N.E. Raine, Combined pesticide exposure severely affects individual- and colony-level traits in bees, Nature. 491 (2012) 105–108, https://doi.org/10.1038/nature11585.

[63] S. Tosi, J.C. Nieh, A common neonicotinoid pesticide, thiamethoxam, alters honey bee activity, motor functions, and movement to light, Sci. Rep. 7 (2017) 1–13, https://doi.org/10.1038/s41598-017-15308-6.

[64] F. Muth, R.L. Gaxiola, A.S. Leonard, No evidence for neonicotinoid preferences in the bumblebee *Bombus impatiens*, R. Soc. Open Sci. 7 (2020), https://doi.org/10.1098/rsos.191883.

[65] H.M. Thompson, S. Wilkins, S. Harkin, S. Milner, K.F.A. Walters, Neonicotinoids and bumblebees (*Bombus terrestris*): effects on nectar consumption in individual workers, Pest Manag. Sci. 71 (2015) 946–950, https://doi.org/10.1002/ps.3868.

[66] C. Alaux, F. Ducloz, D. Crauser, Y. Le Conte, Diet effects on honeybee immunocompetence, Biol. Lett. (2010) 1–4, https://doi.org/10.1098/rsbl.2009.0986.

[67] N. Roger, D. Michez, R. Wattiez, C. Sheridan, M. Vanderplanck, Diet effects on bumblebee health, J. Insect Physiol. 96 (2017) 128–133, https://doi.org/10.1016/j.jinsphys.2016.11.002.

[68] S. Tosi, J.C. Nieh, F. Sgolastra, R. Cabbri, P. Medrzycki, Neonicotinoid pesticides and nutritional stress synergistically reduce survival in honey bees, Proc. R. Soc. B Biol. Sci. 284 (2017), https://doi.org/10.1098/rspb.2017.1711.

[69] J. Tasei, C. Moinard, L. Moreau, B. Himpens, S. Guyonnaud, J. Tasei, C. Moinard, L. Moreau, B. Himpens, S. Guyonnaud, Relationship between aging, mating and sperm production in captive *Bombus terrestris*, J. Apic. Res. 37 (1998) 107–113, https://doi.org/10.1080/00218839.1998.11100962.

[70] R.A. Schwenke, B.P. Lazzaro, M.F. Wolfner, Reproduction–immunity trade-offs in insects, Annu. Rev. Entomol. 61 (2016) 239–256.

[71] B.C. Sheldon, S. Verhulst, B. Sheldon, Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology Reproductive trade-offs, Trends Ecol. Evol. 11 (1996) 317–321.

[72] B. Baer, Bumblebees as model organisms to study male sexual selection in social insects, Behav. Ecol. Sociobiol. 54 (2003) 521–533, https://doi.org/10.1007/s00265-003-0673-5.

[73] V. Dietemann, H.Q. Zheng, C. Hepburn, H.R. Hepburn, S.H. Jin, R.M. Crewe, S. E. Radloff, F.L. Hu, C.W.W. Pirk, Self assessment in insects: honeybee queens know their own strength, PLoS One 3 (2008) e1412, https://doi.org/10.1371/journal.pone.0001412.

[74] P.-F. Röseler, Die Anzahl der Spermien im Receptaculum Seminis von Hummelköniginnen (Hym., Apoidea, Bombinae), Apidologie 4 (1973) 267–274.

[75] J. Kindl, O. Hovorka, K. Urbanová, I. Valterová, Scent marking in male premating behavior of *Bombus confusus*, J. Chem. Ecol. 25 (1999) 1489–1500, https://doi.org/10.1023/A:1020824529729.
[76] M.R. Amin, K.K. Than, Y.J. Kwon, Mating status of bumblebees, *Bombus terrestris* (Hymenoptera: apidae) with notes on ambient temperature, age and virginity, Appl. Entomol. Zool. 45 (2010) 363–367, https://doi.org/10.1303/aez.2010.363.
[77] S. Boff, A. Friedel, R.M. Mussury, P.R. Lenis, J. Raizer, Changes in social behavior are induced by pesticide ingestion in a Neotropical stingless bee, Ecotoxicol. Environ. Saf. 164 (2018) 548–553, https://doi.org/10.1016/j.ecoenv.2018.08.061.
[78] Y. Snellings, B. Herrera, B. Wildemann, M. Beelen, L. Zwarts, T. Wenseleers, P. Callaerts, The role of cuticular hydrocarbons in mate recognition in *Drosophila suzukii*, Sci. Rep. 8 (2018) 1–11, https://doi.org/10.1038/s41598-018-23189-6.
[79] S. Morakchi, A. Maïza, P. Farine, N. Aribi, N. Soltani, Effects of a neonicotinoid insecticide (acetamiprid) on acetylcholinesterase activity and cuticular hydrocarbons profil in German cockroaches, Commun. Agric. Appl. Biol. Sci. 70 (2005) 843–848.
[80] T. Müller, A. Prosche, C. Müller, Sublethal insecticide exposure affects reproduction, chemical phenotype as well as offspring development and antennae symmetry of a leaf beetle, Environ. Pollut. 230 (2017) 709–717, https://doi.org/10.1016/j.envpol.2017.07.018.
[81] I. Valterová, B. Martinet, D. Michez, P. Rasmont, N. Brasero, Sexual attraction: a review of bumblebee male pheromones, Zeitschrift Fur Naturforsch. - Sect. C J. Biosci. 74 (2019) 233–250, https://doi.org/10.1515/znc-2019-0003.
[82] S.E. Crawley, K.A. Kowles, J.R. Gordon, M.F. Potter, K.F. Haynes, Behavioral effects of sublethal exposure to a combination of β-cyfluthrin and imidacloprid in the bed bug, *Cimex lectularius* L, Pest Manag. Sci. 73 (2017) 598–603, https://doi.org/10.1002/ps.4342.
[83] M. Giurfa, Cognition with few neurons: higher-order learning in insects, Trends Neurosci. 36 (2013) 285–294, https://doi.org/10.1016/j.tins.2012.12.011.
[84] D.B. Smith, A.N. Arce, A.R. Rodrigues, P.H. Bischoff, D. Burris, F. Ahmed, R. J. Gill, Insecticide exposure during brood or early-adult development reduces brain growth and impairs adult learning in bumblebees, Proc. R. Soc. B Biol. Sci. 287 (2020), https://doi.org/10.1098/rspb.2019.2442.
[85] S. Piiroinen, D. Goulson, Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees, Proc. R. Soc. B Biol. Sci. 283 (2016), 20160246, https://doi.org/10.1098/rspb.2016.0246.
[86] N. Forfert, A. Troxler, G. Retschnig, L. Gauthier, L. Straub, R.F.A. Moritz, P. Neumann, G.R. Williams, Neonicotinoid pesticides can reduce honeybee colony genetic diversity, PLoS One 12 (2017) 1–14, https://doi.org/10.1371/journal.pone.0186109.
[87] R.E. Snodgrass, The Anatomy of the Honey Bee, Hardpress Publishing, 1962.
[88] A. Ciereszko, J. Wilde, G.J. Dietrich, M. Siuda, B. Bąk, S. Judycka, H. Karol, Sperm parameters of honeybee drones exposed to imidacloprid, Apidologie 48 (2017) 211–222, https://doi.org/10.1007/s13592-016-0466-2.
[89] L. Straub, L. Villamar-bouza, S. Bruckner, P. Chantawannakul, L. Gauthier, K. Khongphinitbunjong, G. Retschnig, A. Troxler, B. Vidondo, P. Neumann, G. R. Williams, L. Straub, Neonicotinoid insecticides can serve as inadvertent insect contraceptives, R. Soc. Proc. B. 283 (2016), 20160506, https://doi.org/10.1098/rspb.2016.0506.
[90] R.J. Paxton, Male mating behaviour and mating systems of bees: an overview, Apidologie. 36 (2005) 145–156, https://doi.org/10.1051/apido:2005007.
[91] F. Ben Abdelkader, G. Kairo, M. Bonnet, N. Barbouche, L.P. Belzunces, J. L. Brunet, Effects of clothianidin on antioxidant enzyme activities and malondialdehyde level in honey bee drone semen, J. Apic. Res. 58 (2019) 740–745, https://doi.org/10.1080/00218839.2019.1655182.
[92] R. Page, Sperm utilization in social insects, Annu. Rev. Entomol. 31 (1986) 297–320, https://doi.org/10.1146/annurev.ento.31.1.297.
[93] J.J. Boomsma, B. Baer, J. Heinze, The evolution of male traits in social insects, Annu. Rev. Entomol. 50 (2005) 395–420, https://doi.org/10.1146/annurev.ento.50.071803.130416.
[94] B. Baer, S.A.O. Armitage, J.J. Boomsma, Sperm storage induces an immunity cost in ants, Nature 441 (2006) 872–875, https://doi.org/10.1038/nature04698.
[95] A. McAfee, A. Chapman, J.S. Pettis, L.J. Foster, D.R. Tarpy, Trade-offs between sperm viability and immune protein expression in honey bee queens (*Apis mellifera*), Commun. Biol. 4 (2021) 1–11, https://doi.org/10.1038/s42003-020-01586-w.
[96] E.A.D. Mitchell, B. Mulhauser, M. Mulot, A. Mutabazi, G. Glauser, A. Aebi, A worldwide survey of neonicotinoids in honey, Science 358 (6359) (2017) 109–111, https://doi.org/10.1126/science.aan3684.
[97] K. Sander, Fertilization and egg cell activation in insects. Biol. Fertil., 2012, pp. 409–430.
[98] D. Schläppi, N. Kettler, L. Straub, G. Glauser, P. Neumann, Long-term effects of neonicotinoid insecticides on ants, Commun. Biol. 3 (2020) 1–9, https://doi.org/10.1038/s42003-020-1066-2.
[99] L. Straub, V. Strobl, P. Neumann, The need for an evolutionary approach to ecotoxicology, Nat. Ecol. Evol. 4 (2020), https://doi.org/10.1038/s41559-020-1194-6.
[100] A. Van Oystaeyen, B.K. Klatt, C. Petit, N. Lenaerts, F. Wäckers, Short-term lab assessments and microcolonies are insufficient for the risk assessment of insecticides for bees, Chemosphere (2020), https://doi.org/10.1016/j.chemosphere.2020.128518.