Title

Field-realistic concentrations of copper but not cadmium reduce survival without affecting reproductive traits in *Bombus terrestris* males

Journal

Environmental Pollution

DOI

10.1016/j.envpol.2025.127080

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Acknowledgment

We would like to thank two anonymous reviewers whose comments greatly improved the quality of the manuscript. A.G. is supported by an F.R.S.-FNRS PhD grant “Aspirant”. M.G. is supported by a postdoctoral grant “Chargé de Recherches” from the F.R.S.-FNRS.

Conflict of interest

No conflict of interest to declare.

Abstract

Understanding how environmental pollutants affect male reproductive traits in bees is critical for elucidating mechanisms underlying pollinator decline. While trace metals are increasingly recognised as widespread contaminants, their effects on male reproductive success in bees remain unexplored. Here, we investigated the impact of field-realistic exposure to copper and cadmium, two prevalent trace metals, on reproductive traits in adult *Bombus terrestris* males. Males were exposed via sucrose solution to copper, cadmium, or a copper–cadmium mixture, and evaluated for survival, competitive mating success, sperm count and sperm viability. Twelve-day exposure to copper and the copper–cadmium mixture significantly increased male mortality (i.e., 82% and 75% mortality, respectively), suggesting physiological stress potentially linked to impaired nutrient assimilation, as evidenced by reduced syrup intake. By contrast, cadmium-exposed males exhibited survival (i.e., 15% mortality) and feeding rates (i.e., 1.65 g of sucrose syrup) comparable to controls and showed no differences in competitive mating success (i.e., 50% of control and 50% of cadmium-exposed males succeeded) or mating duration (i.e., ~29 min for control and cadmium-exposed males). Additionally, six-day exposure to metals did not significantly affect sperm count or viability (i.e., ~63% viability in all treatments), though both parameters showed high inter-individual variability. Our results therefore indicate that adult exposure to field-realistic concentrations of copper and cadmium does not impair reproductive traits in bumble bee males, although copper poses a lethal risk. The absence of detectable effects on sperm quality may reflect limited metal accumulation in reproductive tissues, potentially due to sequestration in other organs. As spermatogenesis starts during larval development in bumble bees, we advocate future studies to also evaluate sperm parameters after larval exposure. Additionally, we encourage further research on additional reproductive traits such as pheromone signalling and sperm transfer efficiency.

Keywords

63 Buff-tailed bumblebee; Heavy metals; Reproduction; Reproductive Competition; Spermatozoa

Introduction

The global decline of bee populations poses a significant threat to food security, particularly as human societies become increasingly dependent on entomophilous crops (Aizen *et al.* 2019; Schulp *et al.* 2014). While the impacts of anthropogenic drivers on bee decline, such as chemical pollution and habitat loss, have been well documented (Guzman *et al.* 2024; Scheper *et al.* 2014; Woodcock *et al.* 2016), the mechanistic pathways through which these stressors reduce bee reproductive success (i.e., underlying mechanisms) remain poorly understood (Gekière *et al.* 2025). Most existing studies focus on lethal and sublethal effects of these pressures, such as the disruption of fat body function following pesticide exposure (Conceição de Assis *et al.* 2022), without addressing how such physiological impairments ultimately impact reproductive success.

In studies of bee population decline, male reproductive success, namely their ability to locate and successfully mate with females, is often overlooked (Belsky *et al.* 2020). In most species, male mating behaviour includes a sequence of activities, namely patrolling, competition with rival males, copulation, sperm transfer, and post-copulatory guarding behaviour (Paxton 2005). Emerging evidence indicates that anthropogenic stressors can impair male mating behaviour and sperm quality (Zhao *et al.* 2021). For example, exposure to heat stress and pesticides has been shown to reduce sperm viability (i.e., the proportion of living spermatozoa) in the reproductive tracts of bumble bee males (Martinet *et al.* 2021; Minnameyer *et al.* 2021). Moreover, pesticide-exposed males initiate copulation with queens 40% faster than unexposed males, suggesting an abnormal eagerness to mate that may reflect altered behavioural regulation (Straub *et al.* 2022). In a recent competitive mating experiment, in which treated and untreated males competed for access to the same female, pesticide-treated males were found to be 30 times less likely to initiate copulation with the female (Chen *et al.* 2024). These effects of anthropogenic pressures on bee reproduction are particularly concerning for

89 monandrous species (i.e., species in which females mate only once), meaning that any reduction
90 in male reproductive success or sperm quality could entirely compromise female reproductive
91 success.

92 Despite the growing body of research on bee males' reproductive fitness, the influence
93 of trace metals on these parameters remains unexplored. Trace metal exposure has gained
94 increasing attention over the past decade due to its pervasive effects on bee health, spanning
95 from community-level disruptions to molecular alterations (Gekière *et al.* 2023). Trace metal
96 pollution primarily originates from fossil fuel combustion, mining operations, agrochemical
97 applications, and household wastewater (Wuana & Okieimen 2011). Additionally, the
98 expansion of metal-intensive renewable energy technologies is projected to substantially
99 increase global metal demand in the coming decades (Sonter *et al.* 2020). In female bees,
100 chronic exposure to trace metals via contaminated food sources or nesting substrates has been
101 associated with impaired reproductive success, including reduced offspring production (Moroń
102 *et al.* 2012; Scott *et al.* 2022). In contrast, the potential impact of trace metals on male mating
103 behaviour and sperm quality in bees remains virtually unexplored. However, evidence from
104 other taxa suggests this is a critical research gap. For instance, in the amphibian *Strauchbufo*
105 *raddei*, metal exposure alters male sexually selected traits and influences mate choice (Su *et al.*
106 2023). In addition, in the tenebrionid beetle *Blaps polycresta*, males collected from
107 contaminated sites exhibited high cadmium accumulation in the testes and a complete absence
108 of spermatozoa (Shonouda & Osman 2018). In humans as well, the detrimental effects of trace
109 metals on sperm parameters are well-documented (López-Botella *et al.* 2021). Together, these
110 findings underscore the need to investigate the impacts of trace metal exposure on male
111 reproductive success in bees.

112 To address this knowledge gap, we investigated the effects of trace metal exposure on
113 male reproductive success in the monandrous buff-tailed bumble bee *Bombus terrestris*, a

widely used model pollinator species. Following emergence, bumble bee males remain in the nest for ~12 days until sexual maturation (Duchateau & Mariën 1995; Minnameyer *et al.* 2021), feeding on potentially contaminated food stores gathered by foragers. After leaving the nest permanently, they rely on floral nectar to meet their energetic demands while competing with other males to engage in mating behaviours (Belsky *et al.* 2020). Here, males were exposed to two prevalent trace metal pollutants, namely copper and cadmium, at field-realistic concentrations during their sexual maturation. In the first experiment, we assessed the impact of metal exposure on male mating performance by placing treated and untreated males in direct competition for access to a single female. By measuring mating success, latency and duration, we assessed whether treated males could succeed in a competitive context and, if so, whether their copulatory behaviour was altered. In a second experiment, we examined the effects of metal exposure on sperm quality, specifically evaluating sperm count and sperm viability, which are crucial parameters in monandrous species. Together, these experiments provide new insights regarding the effects of trace metal contamination on the reproductive success of male pollinators.

Materials and methods

Queen and male maintenance

Nine standard colonies of *Bombus terrestris* L. were obtained from the commercial supplier Biobest (Westerlo, Belgium). Colonies were maintained for approximately two months under laboratory conditions and fed *ad libitum* with Biogluc® sugar solution and *Salix* sp. pollen (Ruchers de Lorraine, France) to promote queen development and emergence. Queen emergence was monitored daily. Newly emerged queens were collected and housed in plastic boxes (10 × 10 × 16 cm; one box per colony per day), with continuous access to a 50% sucrose solution (sucrose:mineral water 1:1 w/w). Daily collection allowed for precise age tracking, ensuring that queens used in subsequent experiments were within the 1–11 day post-emergence

window required for successful mating (Tasei *et al.* 1998). Males of unknown age (Biobest; pers. comm.) were sourced from Biobest Masculino systems, from ten boxes for mating trials (~25 males per box) and six boxes (~15 males per box) for sperm quality assessments. Males were retrieved randomly from various boxes to prevent genetic and age biases. Within their commercial boxes, males were provided *ad libitum* with cotton sticks soaked in 50% sucrose solution. All bees were maintained in a dark room at a constant temperature of 27 ± 1 °C and relative humidity of 60 ± 10% throughout the experimental period.

Treatment

Metal solutions were prepared by dissolving either copper ($CuCl_2$; Sigma-Aldrich, CAS 7447-39-4), cadmium ($CdCl_2$; Sigma-Aldrich, CAS 10108-64-2), or a combination of both in a 50% sucrose solution. Final metal concentrations were quantified at the Mineral and Organic Chemical Analysis (MOCA) platform (Louvain-la-Neuve, Belgium) using an Inductively Coupled Plasma – Optical Emission Spectrometer (ICP-OES; Agilent 5800 VDV). The copper solution contained 41.7 $mg.L^{-1}$ of copper, the cadmium solution contained 0.35 $mg.L^{-1}$ of cadmium, and the mixed-metal solution contained 42.5 $mg.L^{-1}$ of copper and 0.37 $mg.L^{-1}$ of cadmium. The control solution consisted of 50% sucrose without added metals. These concentrations were based on concentrations found in the nectar of melliferous plants (for copper: 42 – 61 $mg.L^{-1}$) (Xun *et al.* 2018) or in pollen stored in bumblebee hives (for cadmium: 0.02 $mg.L^{-1}$) (Sivakoff *et al.* 2020).

Experiment 1 – Competitive mating

Males were individually housed in Nicot cages and provided *ad libitum* access to their respective treatment via 2-mL tipless syringes for a period of 12 days ($n \approx 120$ for the control group and $n \approx 40$ for each metal-exposed group). This exposure duration corresponds to the full period of sexual maturation, as *Bombus terrestris* males typically reach mating competency around 12 days of age, with complete sperm migration to the accessory testes occurring by day

13, and remain sexually mature until death (Duchateau & Mariën 1995; Minnameyer *et al.* 2021). Therefore, although the males' age at receipt was unknown, we were confident that all males were sexually mature after the exposure period. Syrup consumption was monitored by weighing each syringe at the start of the experiment, reweighing and refilling on day 6, and weighing again at the end of the exposure period. All individuals consumed their respective solutions, indicating that no complete avoidance occurred. Male mortality was recorded daily.

Following exposure, surviving males were individually marked on the scutum using water-based markers, assigning distinct colours to control and metal-treated groups. Mating trials were conducted in a 30 × 30 × 30 cm flight arena, where one control male and one treated male were introduced simultaneously and allowed a 10-min acclimation period. Pairs of males were selected randomly but were from different commercial boxes to avoid sibling bias. Subsequently, a sexually mature queen was introduced into the arena for 50 min. If no mating occurred, the queen was replaced with a new queen for an additional 50-min observation period. Out of 53 competitive mating trials, only five resulted in no copulation after the second queen was introduced. These unsuccessful matings were not included in further analyses.

For each competitive mating trial, we recorded: (i) the group of the successful male (i.e., control or treated), (ii) mating latency (i.e., time from queen introduction to copulation onset) and (iii) mating duration (i.e., time the male and queen remained attached). After the mating period, males were frozen and weighed.

Experiment 2 – Sperm quality assessment

Males were housed individually and provided *ad libitum* access to their respective treatment, following the same protocol as described in Experiment 1 ($n \approx 20$ for control and each metal-exposed group). However, the exposure duration was limited to six days, as prolonged exposure for 12 days led to significant mortality (see Results section). Although this exposure period may not allow for full sexual maturation, males have been reported to successfully mate from six

189 days of age onwards (Tasei *et al.* 1998). We were therefore confident that spermatozoa were
190 present in the testes during the exposure period. At the end of the exposure period, males were
191 anaesthetised by asphyxiation in 2-mL centrifuge tubes. Each individual was then pinned to a
192 foam surface, and the abdomen was opened using dissecting scissors by making lateral incisions
193 between the tergites and sternites. The entire reproductive tract, including the testes, accessory
194 testes, accessory glands, ejaculatory duct, endophallus, and genitalia, was excised with fine
195 forceps and transferred into 100 µL of Ringer's solution (i.e., NaCl 9 g, KCl 0.2 g, $NaHCO_3$ 0.2
196 g, $CaCl_2$ 0.2 g in 1 L of distilled water) in a 1.5-mL centrifuge tube. Homogenisation of the
197 reproductive tract was achieved by applying five steady, non-rotating crushing motions. The
198 genitalia and crushed tissues were then removed from the solution using tweezers. The resulting
199 suspension was split into two equal volumes: 50 µL were used for sperm count and 50 µL were
200 allocated for sperm viability assessment.

201 For sperm count, samples were first diluted five times in Ringer's solution to facilitate
202 counting. Three 1-µL drops of the diluted sperm suspension were placed on a microscope slide
203 and air-dried under a chemical hood. Once dry, the spermatozoa were fixed using RAL Diff-
204 Quik™ Fixative Solution (RAL Diagnostics, Martillac, France) and air-dried again. Slides were
205 then stained by sequential immersion: five 1-sec dips in RAL Diff-Quik™ Solution I, followed
206 by draining, five 1-sec dips in RAL Diff-Quik™ Solution II, and rinsing with tap water. After
207 a final air-drying step, slides were mounted using ROTI® Histokitt mounting medium (Carl
208 Roth; Karlsruhe, Germany) and sealed with a cover glass, which was left to dry overnight under
209 the flow hood. Each stained drop was divided into four equal quadrants, and the number of
210 spermatozoa in the upper-left quadrant was counted under a light microscope at 400x
211 magnification (Motic® BA210LED; Motic Europe, Barcelona, Spain). The mean number of
212 spermatozoa from the three drops was used as the sperm count for each individual male.

For sperm viability assessment, 1 µL of propidium iodide (PI; 1 mg.mL^{-1}; Sigma-Aldrich, CAS 25535-16-4) and 0.5 µL of Hoechst 33342 (0.5 mg.mL^{-1}; Sigma-Aldrich, CAS 23491-52-3) were added to each sperm suspension. Samples were incubated for 20 min in the dark at room temperature (Wegener *et al.* 2012). Following incubation, 8 µL of the stained suspension were placed on a microscope slide and examined under a fluorescence microscope at 400x magnification (Nikon® Eclipse Ti2-U; Nikon Corporation, Tokyo, Japan), equipped with DAPI and mCherry filter cubes. Hoechst staining was used to label all sperm nuclei, while PI selectively stained only non-viable cells. For each sample, 100 spermatozoa were counted, and the proportion of non-viable spermatozoa (i.e., PI-stained cells) was recorded. Sperm viability was then expressed as the proportion of viable spermatozoa, calculated as 1 – proportion of dead spermatozoa.

Statistical analyses

Survival during the exposure phase and mating success during the reproductive phase were analysed using Cox proportional hazards regression models implemented in the *survival* package (Therneau 2021), with treatment as a fixed effect for survival analyses and treatment as well as male mass as fixed effects for mating success analyses. Individuals who experienced the event of interest, death (for survival analysis) or copulation (for mating success), were treated as uncensored. We used Cox proportional hazards models rather than log-rank tests because Cox models allow estimation of hazard ratios between treatments and the inclusion of continuous covariates (i.e., male mass). Proportional hazards assumptions were confirmed ($p > 0.05$) for each model using diagnostic tools provided by the *survival* package (Therneau 2021).

Syrup consumption was assessed only during the first six days of exposure, using live individuals at day six, as high mortality in some treatments by day 12 precluded reliable measurements at the end of the exposure period. Syrup consumption and mating duration were analysed using linear models (LMs) with treatment as a fixed effect for syrup consumption

analyses and treatment as well as male mass as fixed effects for mating duration analyses, using the *stats* package (R Core Team 2020). The assumptions of normality and homoscedasticity of residuals were visually assessed using diagnostic plots generated with the *ggfortify* package (Tang *et al.* 2016) (**Appendix 1** for syrup consumption; **Appendix 2** for mating duration).

Sperm count, treated as a count variable, was analysed using a generalised linear model (GLM) with a negative binomial distribution and a log link. Sperm viability, expressed as a proportion bounded between 0 and 1, was analysed using a GLM with a beta distribution and a logit link. Both models were fitted with treatment as a fixed effect using appropriate family specifications using the *glmmTMB* package (Brooks *et al.* 2017). Model fits were assessed visually using the *DHARMa* package (Hartig 2021) (**Appendix 3** for sperm count; **Appendix 4** for sperm viability).

Statistical significance of model terms was evaluated using Type-II analysis of variance tables from the *car* package (Fox *et al.* 2019). When a significant treatment effect was detected ($p < 0.05$), *post hoc* pairwise comparisons were performed to compare each treatment group against the control, using the *emmeans* package with false discovery rate (FDR) correction for multiple testing (Lenth 2022).

Data visualisations, including Kaplan–Meier survival curves and boxplots, were produced using the *survminer* (Kassambara *et al.* 2021) and *ggplot2* packages (Wickham *et al.* 2020). All statistical analyses were performed in R version 4.4.0 (R Core Team 2024).

Results

Experiment 1 – Competitive mating

Bumble bee males were exposed for 12 days to field-realistic concentrations of trace metals. Exposure resulted in significantly increased mortality ($\chi^2 = 126.6$, df = 3, $p < 0.001$). Custom contrast analyses revealed that males exposed to copper or the mixed-metal solution were approximately 17 times more likely to die than those in the control group (HR for copper =

17.73; HR for mixed-metal = 17.18). In contrast, mortality in the cadmium treatment did not significantly differ from the control (**Figure 1**). Of the 40 males initially exposed in each of the copper and mixed-metal treatments, only seven and ten individuals, respectively, survived the exposure period. Due to insufficient surviving individuals, these two treatments were excluded from the statistical analysis of competitive mating outcomes. Additionally, syrup consumption was only analysed during the first six days of the experiment, as mortality rates in the copper and mixed-metal treatments were too high after 12 days to allow meaningful comparisons.

A total of 34 cadmium-exposed males were included in the competitive mating trials against control males. Exposure to field-realistic concentrations of cadmium had no significant effect on mating success; exactly half of the successful matings (17 out of 34) were achieved by cadmium-treated males, and the other half by control males ($\chi^2 = 0.022$, df = 1, p = 0.88; **Figure 2**). Likewise, male mass did not influence mating success ($\chi^2 = 0.075$, df = 1, p = 0.78). Similarly, mating duration did not differ between treatments (F = 0.013, df = 1, p = 0.91; **Figure 3**) or according to male mass (F = 0.033, df = 1, p = 0.86). On average, mating lasted 29 min in both groups (mean ± SD: 28.5 ± 8.15 min for control males; 28.6 ± 8.30 min for cadmium-treated males).

Syrup consumption during the first six days of the experiment, measured from live individuals on day six, differed significantly among treatments (F = 21.66, df = 3, p < 0.001; **Figure 4**). Custom contrast analyses revealed that males exposed to copper (mean ± SD: 1.34 ± 0.29 g) or the mixed-metal solution (mean ± SD: 1.26 ± 0.23 g) consumed significantly less syrup than control males (mean ± SD: 1.65 ± 0.28 g). In contrast, syrup consumption by cadmium-treated males (mean ± SD: 1.65 ± 0.36 g) did not differ from that of the control group.

Experiment 2 – Sperm quality assessment

Bumble bee males were exposed for six days to field-realistic concentrations of trace metals. Exposure had no significant effect on sperm count ($\chi^2 = 4.89$, df = 3, p = 0.18; **Figure 5A**).

Similarly, sperm viability was not significantly affected by metal exposure ($\chi^2 = 4.75$, df = 3, p = 0.19; **Figure 5B**). Mean sperm viability was 61 ± 22% in the control group, 60 ± 25% in the cadmium treatment, 58 ± 25% in the copper treatment, and 73 ± 17% in the mixed-metal treatment (mean ± SD), indicating substantial inter-individual variability but no clear pattern between treatments.

Discussion

This study provides the first investigation into the effects of field-realistic concentrations of trace metals on the reproductive fitness of males in a major pollinating species. Contrary to our expectations, 12-day exposure to cadmium had no discernible impact on the males' ability to successfully compete with untreated rivals for access to a female, and no discernible impact on mating duration. By contrast, prolonged exposure for 12 days to copper or the copper–cadmium mixture resulted in increased male mortality. On the other hand, 6-day chronic exposure to copper, cadmium, or their combination did not affect sperm quality (i.e., sperm count and viability) in bumble bee males.

The increased mortality observed in males exposed to copper or the copper–cadmium mixture for 12 days, in contrast to the absence of mortality effects from cadmium alone, is consistent with findings from previous studies. For instance, honey bee workers exposed to copper concentrations comparable to those used in the present study (i.e., field-realistic) exhibited over 20% mortality within three days, whereas cadmium had to be applied at concentrations approximately 100 times higher than ours (i.e., not field-realistic) to induce similar mortality levels (Di *et al.* 2016). Similarly, bumble bee workers showed significant mortality following chronic exposure to copper at the concentrations used here, while cadmium needed to be applied at three times the tested concentration to elicit comparable effects over a seven-day period (Rothman *et al.* 2020). The increased mortality observed in males exposed to copper or the copper–cadmium mixture is likely attributable to a physiological disruption

caused by metal-induced redox imbalance and associated macromolecular damage (Valko *et al.* 2005). Such damage also occurs in the gut epithelium (e.g., Bernardes *et al.* 2021), which may exacerbate the physiological toxicity of metals by impairing digestive function and reducing feeding motivation, as indicated by the decreased syrup consumption observed in exposed males. This reduced intake may further compromise detoxification processes by limiting the energetic resources required for effective metal clearance. We are confident that the observed mortality resulted from metal-induced physiological disruption rather than starvation due to avoidance of metal-laced sucrose, as complete feeding avoidance would likely have led to death within 24 hours in all exposed individuals (Brown *et al.* 2000), which was not observed. In contrast, although cadmium is a non-essential element typically considered more toxic than essential metals such as copper, the concentrations used in this study were 117-fold (for cadmium) and 19-fold (for copper) lower than the median lethal concentrations (LC_{50}) previously reported for workers (Gekière *et al.* 2024). Despite being field-realistic, the copper concentration was therefore relatively closer to its LC_{50} than cadmium, which may explain the observed differences in toxicity. This comparison underscores that essential metals, although required in trace amounts, can pose a greater toxic risk under environmentally relevant conditions. These findings emphasise the importance of evaluating not only the intrinsic hazard of metals but also the risk they pose, determined by both the nature of the metal and the extent of exposure levels (Adriaanse *et al.* 2023).

In addition to having no significant effect on male mortality, prolonged cadmium exposure did not impact the mating success or mating duration of bumble bee males. Males exposed to cadmium for 12 days were equally successful as unexposed males in securing copulations during competitive mating trials and exhibited similar mating durations. Given that close-range olfactory cues emitted by queens trigger copulation attempts in conspecific males (Ayasse & Jarau 2014), our findings suggest that cadmium exposure did not impair male

antennal sensitivity to queen-emitted sexual pheromones. However, it is important to note that this study, like all laboratory-based research on bumble bee males, did not assess the potential effects of xenobiotics on male scent-marking and patrolling behaviours, which are key components of male reproductive strategy in natural environments (Baer 2003). Moreover, this study did not examine multi-male competition or the flight distances required to reach females, which are factors that may pose significant challenges for male bumble bees under natural conditions (Baer 2003). In the wild, bumble bee males establish patrol routes and deposit pheromones from their cephalic labial glands onto vegetation to attract virgin queens (Valterová *et al.* 2019). To date, the impact of pollutants such as pesticides or trace metals on male pheromone production remains largely unexplored, in contrast to some documented effects of thermal stress (Przybyla *et al.* 2021). Moreover, although mating duration was not affected by cadmium exposure, this does not preclude potential effects on sperm transfer efficiency or the biochemical composition of the mating plug, a gelatinous secretion that plays a role in inhibiting queen remating (Baer *et al.* 2001; Duvoisin *et al.* 1999). Future ecotoxicological studies should investigate these parameters, as they are critical components of male reproductive fitness and may be sensitive to environmental pollutants (Belsky *et al.* 2020).

Intriguingly, although exposure to copper and the copper–cadmium mixture resulted in increased male mortality over 12 days of exposure, no significant effects were observed on sperm count or viability after a 6-day exposure. To date, the accumulation of metals in bee tissues remains poorly understood, with most studies investigating metal accumulation primarily in the gut and hepato-nephrocytic tissues (Al-Naggar *et al.* 2013; Nogueira *et al.* 2019). Given the well-documented cytological effects of heavy metals, the absence of any detectable impact on sperm quality in our study suggests that copper and cadmium either did not reach the reproductive tissues of bumble bee males or accumulated in negligible amounts. These findings contrast with a previous study on tenebrionid beetles, which reported significant

363 cadmium accumulation in the testes of males from polluted areas (Shonouda & Osman 2018).
364 Hence, future studies using histological or analytical methods are necessary to confirm whether
365 reproductive tissues are indeed protected from metal accumulation. Our findings contribute to
366 the growing body of evidence indicating that bees may sequester metals in specific tissues prior
367 to excretion, thereby minimising systemic toxicity (Borsuk *et al.* 2021). Similar sequestration
368 mechanisms have been documented in the midgut of lady beetles (Rost-Roszkowska *et al.*
369 2008) and the cuticle of fruit flies (Vásquez-Procopio *et al.* 2020). By shielding reproductive
370 tissues from metal exposure, bees may thus enhance their chances of reproductive success.
371 Although metal exposure during adulthood did not negatively affect sperm quality in bumble
372 bee males, it remains crucial to investigate the effects of exposure during larval development.
373 In bumble bees, spermatogenesis occurs during the larval stage, and males emerge with a fixed
374 number of sperm stored in their testes (Baer 2003). In addition, trace metal exposure during
375 larval development has been shown to reduce adult body size (Di *et al.* 2016), a key determinant
376 of mating success in male bees (Amin *et al.* 2012). As a result, assessing the impact of
377 contaminants such as trace metals during this critical developmental period would provide a
378 more comprehensive understanding of their effects on male reproductive success. Finally, while
379 heat stress is known to compromise sperm DNA integrity in male bumble bees (Martinet *et al.*
380 2021), the potential effects of trace metals on this reproductive parameter remain to be
381 investigated.

382 Although no significant effects on sperm quality were detected among treatments, it is
383 noteworthy that both sperm count and viability exhibited substantial within-treatment
384 variability. Specifically, we observed up to a ten-fold variation in sperm count (ranging from
385 fewer than 30 to over 300 spermatozoa) and a three-fold variation in sperm viability (from less
386 than 25% to more than 75%) within individual treatments. Similar variability has been reported
387 in previous studies investigating the effects of pesticides on sperm quality in bumble bees (e.g.,

Straub *et al.* 2016) and solitary bees (e.g., Strobl *et al.* 2021), as well as in studies addressing the effects of heatwaves on sperm quality in bumble bees (e.g., Martinet *et al.* 2021). By contrast, a comparative study across insect taxa reported consistently high sperm viability (i.e., more than 90%) in both honey bee and bumble bee males (Hunter & Birkhead 2002). In light of such discrepancies, we emphasise the need for future studies on bee sperm quality to employ rigorous sample replication, to clearly define and standardise their viability staining methods, and to ensure robust statistical analyses. Moreover, in bumble bees, potential differences in sperm count and viability between commercial and wild individuals, as well as across male age during sexual maturity, remain unexplored.

Conclusion and perspectives

Overall, our findings indicate that field-realistic concentrations of cadmium did not affect the mating success of bumble bee males, and that neither copper nor cadmium exposure significantly affected sperm quality. While these results are encouraging, it is important to note that prolonged exposure to copper significantly increased male mortality, underscoring the potential risk this metal poses to male survival in natural environments.

Importantly, the absence of detectable effects on mating success and sperm quality does not exclude the possibility of impacts on other unmeasured parameters, such as sperm DNA integrity or complex reproductive behaviours like scent-marking and patrolling. Trace metal exposure may also exert epigenetic effects that manifest only in subsequent generations, underscoring the need for transgenerational studies on related colonies to fully capture long-term effects on reproductive success.

Furthermore, since males emerge with a fixed sperm supply and body size, both determined during larval development and strongly linked to mating success, the exclusive focus on adult exposure in most studies may miss critical effects that occur during larval development. Future research should therefore consider larval exposure to anthropogenic

stressors to more accurately assess their impacts on male reproductive success. Overall, all these open questions highlight the importance of investigating male reproductive success as a promising avenue for understanding bee population declines in metal-contaminated environments.

Figure 1. Kaplan-Meier survival curves of bumble bee males exposed for 12 days to field-realistic concentrations of metals. P-value is retrieved from the Cox proportional hazards regression. Asterisks indicate significant differences compared to the control group (custom contrasts).

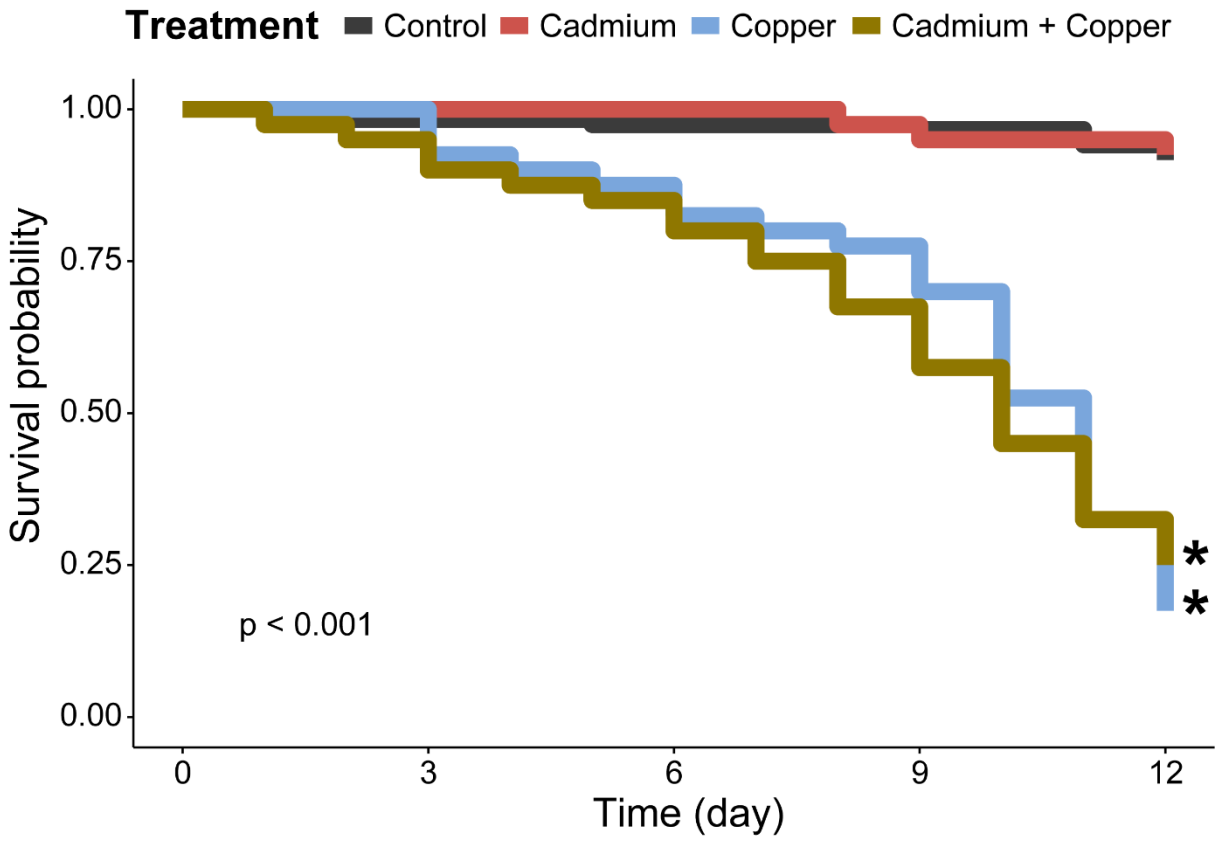


Figure 2. Cumulative incidence of mating between control and cadmium-treated bumble bee males placed in a flight arena with a queen for 50 min. P-value is retrieved from the Cox proportional hazards regression.

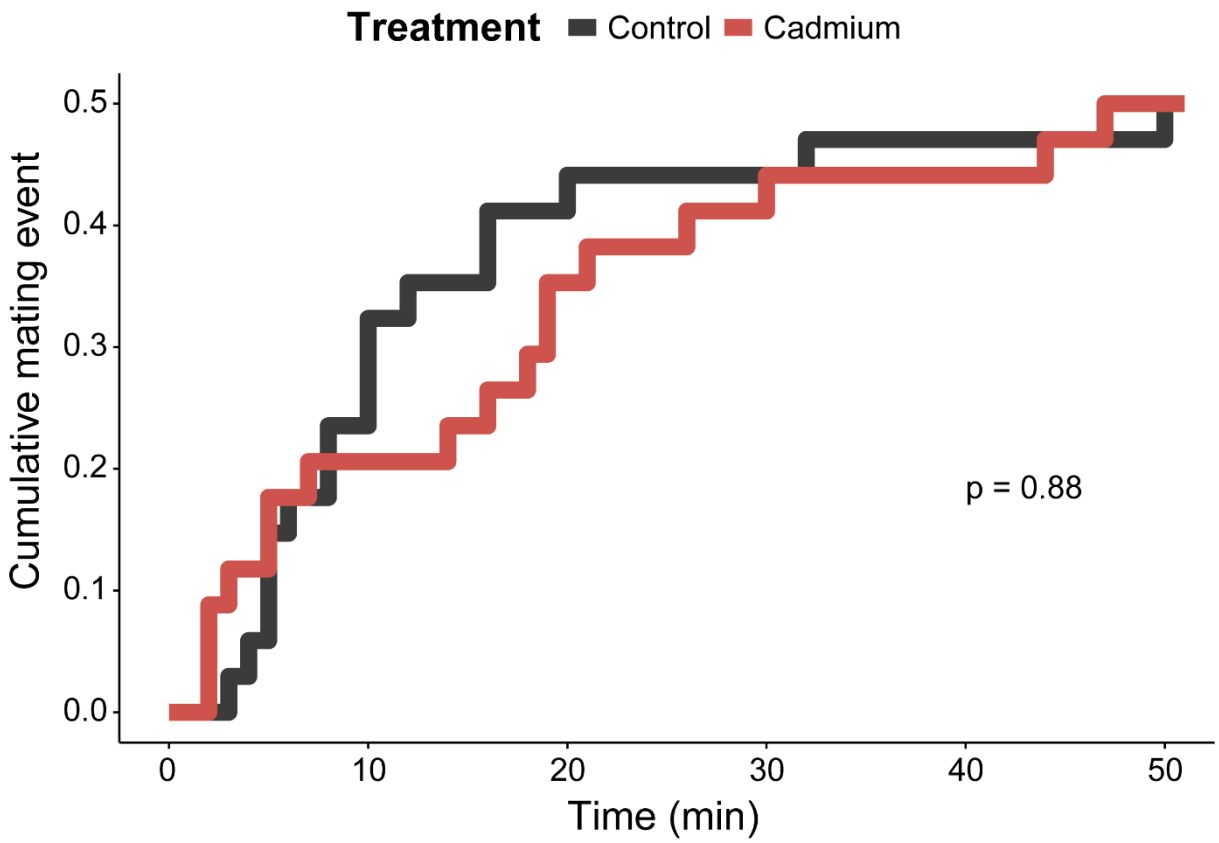


Figure 3. Mating duration between control and cadmium-treated bumble bee males. Large dots and associated lines depict the means and standard errors. P-value is retrieved from the linear model.

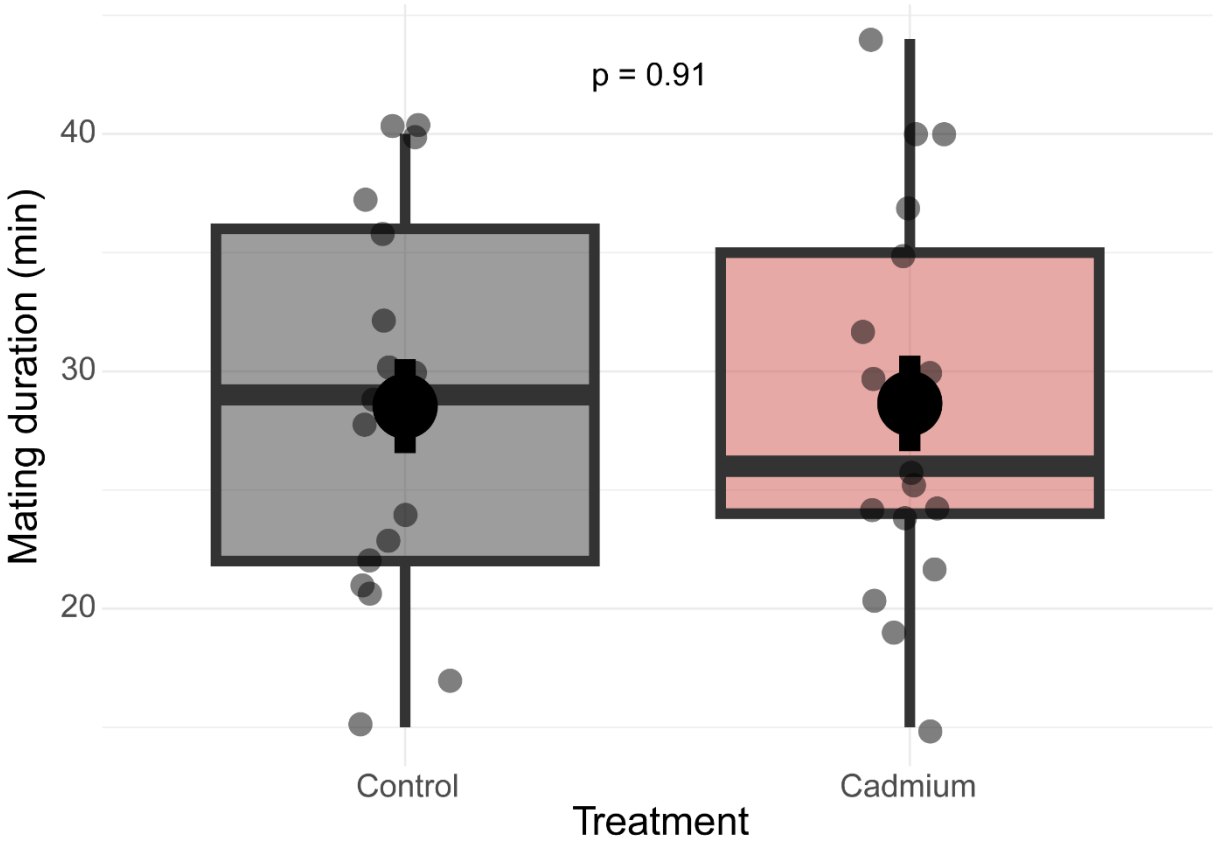


Figure 4. Total syrup consumption of bumble bee males during the first six days of the exposure period. Large dots and associated lines depict the means and standard errors. P-value is retrieved from the linear model. Asterisks indicate significant differences compared to the control group (custom contrasts).

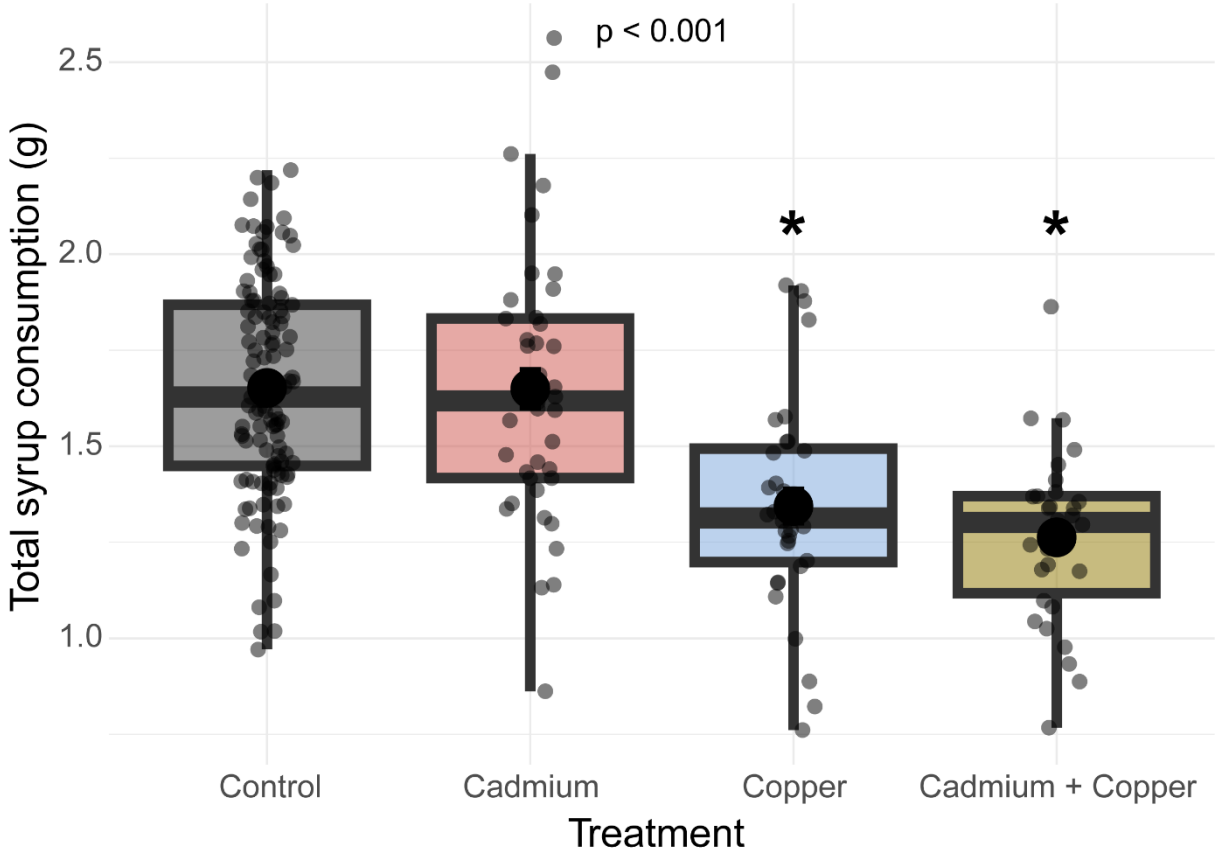
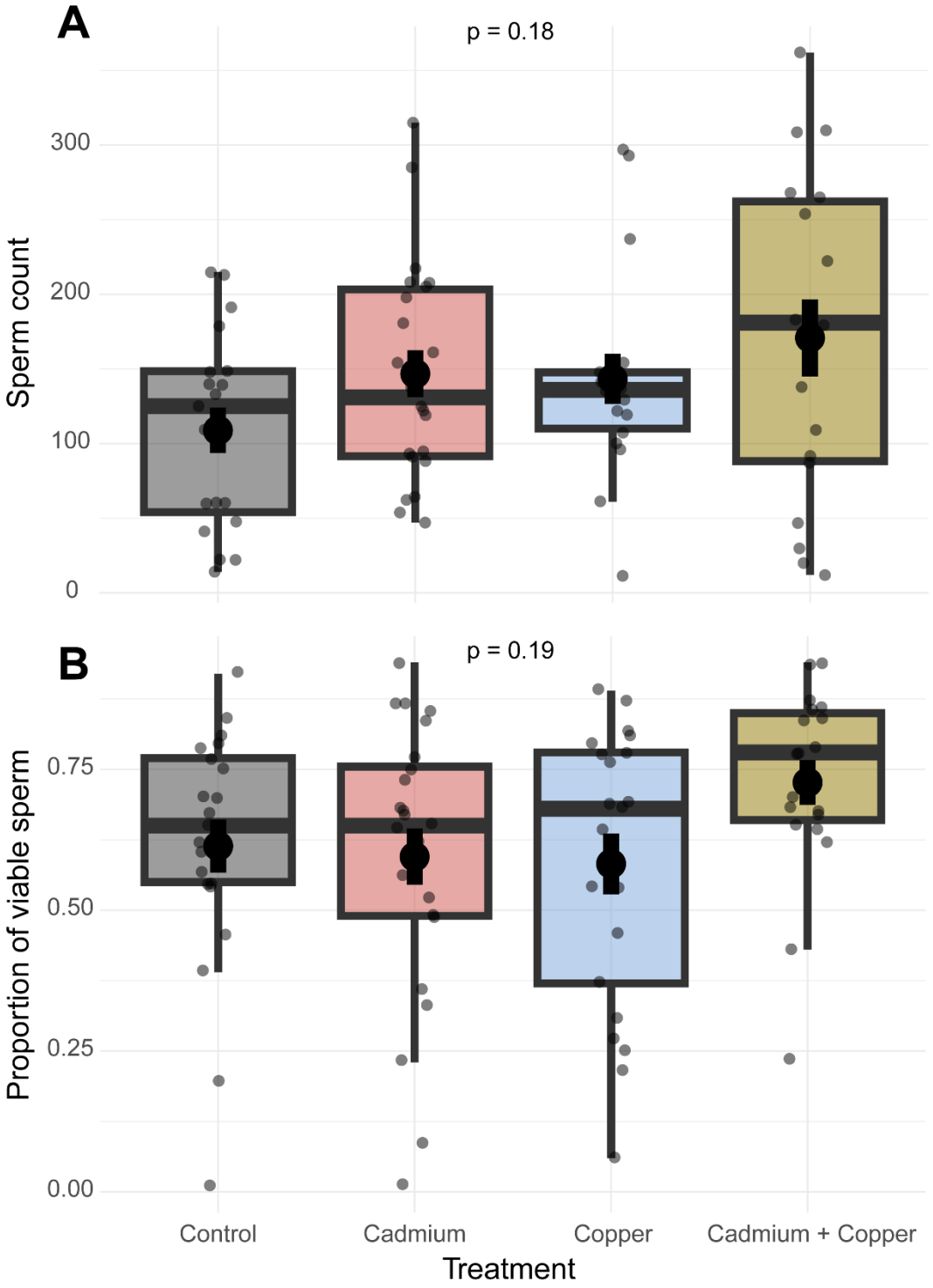


Figure 5. Sperm quality of bumble bee males exposed for 6 days to field-realistic concentrations of metals. **(A)** Sperm count. **(B)** Proportion of viable sperm. P-values are retrieved from generalised linear models.



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