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SPECIAL ISSUE ARTICLE

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Perception, regulation, and fitness effects of pollen phytosterols in the bumble bee, *Bombus terrestris*

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Abstract

Premise: Many flowering plants depend on insects for pollination and thus attract pollinators by offering rewards, mostly nectar and pollen. Bee pollinators rely on pollen as their main nutrient source. Pollen provides all essential micro- and macronutrients including substances that cannot be synthesized by bees themselves, such as sterols, which bees need for processes such as hormone production. Variations in sterol concentrations may consequently affect bee health and reproductive fitness. We therefore hypothesized that (1) these variations in pollen sterols affect longevity and reproduction in bumble bees and (2) can thus be perceived via the bees' antennae before consumption.

Methods: We studied the effect of sterols on longevity and reproduction of *Bombus terrestris* workers in feeding experiments and investigated sterol perception using chemotactile proboscis extension response (PER) conditioning.

Results: Workers could perceive several sterols (cholesterol, cholestenone, desmosterol, stigmasterol, β-sitosterol) via their antennae but not differentiate between them. However, when sterols were presented in pollen, and not as a single compound, the bees were unable to differentiate between pollen differing in sterol content. Additionally, different sterol concentrations in pollen neither affected pollen consumption nor brood development or worker longevity.

Conclusions: Since we used both natural concentrations and concentrations higher than those found in pollen, our results indicate that bumble bees may not need to pay specific attention to pollen sterol content beyond a specific threshold. Naturally encountered concentrations might fully support their sterol requirements and higher concentrations do not seem to have negative effects.

KEYWORDS

Bombus terrestris, feeding assay, microcolonies, nutrition, PER, perception, phytosterols, proboscis extension response conditioning

The majority of flowering plants rely on insects for pollination (Hoshiba and Sasaki, 2008). This mutualistic relationship provides outcrossing for plants and a reward for pollinators (Fægri and Van Der Pijl, 1980). Rewards comprise, e.g., shelter or mating sites but mostly food such as nectar and pollen (Armbruster, 2012). Nectar provides bees with carbohydrates as their primary source of energy, while pollen contains all other important nutrients including proteins and lipids, which are needed by the adults themselves and for rearing brood (Stabler et al., 2015; Grund-Mueller et al., 2020). For an optimal diet, bees further need various micronutrients such as sterols,

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minerals, and vitamins (Roulston et al., 2000; Vanderplanck et al., 2014; Roger et al., 2017), which are also provided by pollen. However, pollen of different plant species and even of different individuals of the same species can vary strongly in its nutritional composition (Roulston et al., 2000; Egan et al., 2018; Ruedenauer et al., 2019b; Eilers et al., 2020; Zu et al., 2021). Depending on pollen quantity and nutrient composition (henceforth referred to as quality), its consumption may thus significantly affect bee foraging behavior, health, and fitness. For example, high amounts of fatty acids or high fatty acid to protein ratios are detrimental to bumble bees (Vaudo et al., 2016; Ruedenauer et al., 2020). With regard to protein, Archer et al. (2021) found that a high amino acid intake increased bumble bee body mass. Similarly, pollen protein content up to 37% positively correlated with offspring size in *Lasioglossum zephyrum*, but a protein content beyond 37% increased offspring mortality rate (Roulston and Cane, 2002). Besides the total amount of specific nutrients, often the ratios (e.g., protein to lipid ratio, P:L) can play an important role. For example, *Bombus impatiens* foragers increase their consumption of pollen with high P:L ratios (Vaudo et al., 2016). These studies show that the effect of nutrients on bee development and fitness are dose- and ratio-dependent. Bees could therefore benefit from being able to assess the quality of pollen by choosing pollen with appropriate nutrient concentrations and ratios to balance their nutrient intake (Ruedenauer et al., 2016; Roger et al., 2017).

Among the different micronutrients, phytosterols (belonging to the group of lipids) are considered of particular importance to insects (Clayton, 1964; Bean, 1973; Svoboda et al., 1978). For example, sterols play a major role in the production of hormones, i.e., ecdysteroids (Clayton, 1964; Robbins et al., 1971; Svoboda et al., 1978; Svoboda and Feldlaufer, 1991), such as 20-hydroxyecdysone, which is the molting hormone in many insect species and requires cholesterol as a precursor. Cholesterol can be obtained by dealkylation of other sterols (Svoboda et al., 1978; Ikekawa et al., 1993). However, some insects, including honey bees, have lost the ability to dealkylate cholesterol (Svoboda et al., 1983, 1994) and therefore use makisterone A as a molting hormone, which is derived from campesterol, or makisterone C derived from sitosterol (Feldlaufer et al., 1985; Jing and Behmer, 2020; Zu et al., 2021). Phytosterols are additionally essential for maintaining the structural integrity of cells and tissues (Clayton, 1960; Gilbert, 1967; Ritter and Wientjens, 1967). For example, 24-methylenecholesterol, β-sitosterol, and δ5-avenasterol affect the size and growth rate of bumble bee larvae (Vanderplanck et al., 2014). However, insects are generally unable to synthesize sterols de novo (Svoboda et al., 1978; Canavoso et al., 2001) and therefore require external sources for their sterol supply. Especially for herbivorous insects, the variation in dietary sterol type and ratio are of vital importance (Behmer, 2017).

Bees rarely collect pollen for themselves, but rather to meet the nutritional requirements of their offspring and, in case of social species like honey bees and bumble bees, also of the whole colony (Brodschneider and Crailsheim, 2010; Nicholls and Hempel de Ibarra 2017; Lihoreau et al., 2018). Consequently, workers respond to colony needs and alter the collection of proteins and lipids when brood is present, as shown for the buffed-tailed bumble bee (*Bombus terrestris*) (Kraus et al., 2019). Unlike nectar, pollen is not ingested by bees during collection (except for a few species, like *Hylaeus* sp.; Daly and Magnacca, 2003), but carry it on their scopa. It is therefore unlikely that foragers evaluate pollen quality via ingestion (Thorp, 2000). To ensure the collection of nutritionally appropriate pollen, they may instead rely on pre-ingestive quality assessment through nutrient perception via receptors on their antennae or tarsi. Most nutrients are not very volatile (Roulston and Cane, 2000; Dijkstra, 2016), and therefore require physical contact for perception. In fact, bumble bees are able to use chemotactile cues to differentiate artificial pollen surrogates that vary in quality, e.g., casein content (Ruedenauer et al., 2015). Moreover, we showed recently that honey bees can perceive amino acids and fatty acids but failed to perceive sterols in pollen via chemotactile antennal stimulation (Ruedenauer et al., 2021). Similarly, bumble bees can perceive amino acids and fatty acids via antennal contact (Ruedenauer et al., 2019a, 2020) but, unlike honey bees, bumble bees cannot differentiate between pollen grains that differ in amino acid content. However, it is still unclear whether bumble bees can perceive sterols, particularly in pollen.

Pollen of different plant species can vary considerably in total sterol concentrations, ranging from 0.12% (*Muscari botryoides*) up to 2.68% of total pollen mass (*Pyrus communis*; Vanderplanck et al., 2019; Zu et al., 2021). Sterols can be found in pollenkitt as well as in internal pollen fractions in almost all plant species analyzed so far (Dobson, 1988). Sterol concentrations were higher in the internal fractions than in pollenkitt, while sterol esters were more abundant in the pollenkitt (Dobson, 1988). However, more detailed information about exact sterol concentration or composition in different pollen parts are still lacking.

In this study, we investigated sterol perception via chemotactile antennal stimulation in *Bombus terrestris* workers. We applied a recently established conditioning assay (Ruedenauer et al., 2015, 2019a), which allows us to test whether nonvolatile nutrients can be perceived via the antennae. In addition, we investigated whether different sterol concentrations in pollen affect longevity and brood development in *B. terrestris* microcolonies using a no-choice feeding experiment with pollen differing in sterol content. Based on recent findings showing that pollen compounds were more likely to be perceived when they have a significant effect on bee health and fitness following over- or underconsumption (Ruedenauer et al., 2020), we hypothesized that (1) increasing sterol concentrations in pollen affects *B. terrestris* colony development and worker longevity and that (2) sterols can therefore be perceived and

differentiated by workers when presented both in isolation and in pollen.

MATERIALS AND METHODS

Animals

The experiments were conducted in two experimental series in 2021. The first series took place from January to April, the second from August to December. In total, 26 commercial *Bombus terrestris* colonies, 14 colonies in experimental series 1 (Behr, Kampen, Germany) and 12 colonies in experimental series 2 (Biobest, Westerlo, Belgium), were bought and transferred to two-chambered wooden nest boxes, comprising a nesting and a foraging chamber (240 × 210 × 110 mm each). All sides of the chambers had holes covered with wire mesh to enhance airflow. The bottom of the box was covered with a thin layer of cat litter (Bio-Catolet, Albbruck, Germany). The colonies were fed ad libitum with polyfloral pollen collected by honey bees from Hungary (Naturwaren Niederrhein GmbH, Goch-Asperden, Germany; for sterol content, see Appendix S1 and Table S1 in Appendix S2) and API-Invert, a mixture of sucrose, fructose, and glucose (Südzucker AG, Mannheim, Germany). The boxes were capped with transparent acrylic lids incorporating holes with a lid for easy access to the colony. All colonies were kept in a climate chamber at 25°C, 60% RH, with a 12 h/12 h light/dark cycle.

Proboscis extension response (PER) experiments

Experimental procedure

We used the differential chemotactile PER conditioning setup established by Ruedenauer et al. (2015). Proboscis extension response conditioning was initially established for honey bees (Bitterman et al., 1983) and is based on the principles of Pavlov's classical conditioning (1928). Bumble bees, like honey bees, show an innate proboscis extension response (i.e., extend their proboscis) when their antennae, tarsi, or parts of their mouth contact a sucrose solution (=unconditioned stimulus [US]). During conditioning, a second stimulus that does not naturally induce PER (i.e., pollen or nutrient to be tested = conditioned stimulus [CS]) is presented simultaneously with the US. If the bee is able to perceive the CS, it will associate the CS with the US. Subsequently, the CS alone can elicit a PER (Laloi et al. 1999). In addition, we can test whether the bees can differentiate between two stimuli (differential conditioning) by associating only one of the two conditioned stimuli with the sucrose solution (rewarded CS = CS+), whereas the second stimulus is presented without reward (unrewarded CS = CS−).

One day before an experiment took place, 30 workers were collected randomly from one colony. The bees were chilled on ice until they were immobilized. They were then carefully put in shortened 1-mL pipette tips and fixed with a "yoke" made from wire and tape, so that the head and forelegs could move freely (Appendix S3, Figure S1). The harnessed bees were then fed with 30% w/w sucrose solution ad libitum and kept in a moist box placed in a climate chamber at 25°C for approximately 19 h.

Before the experiment started, each bumble bee was tested for its motivational state by touching its antennae with a toothpick soaked in 50% w/w sucrose solution. Only those bumble bees that showed a PER were used for further testing. One bee at a time was placed in a forward-tilted rack that allowed easy access to the bee's antennae (Appendix S3, Figure S1) and was allowed to rest for 15 s. Then, the stimulus (CS+ or CS−), applied on a piece of filter paper (5 × 5 mm), was presented on a copper plate, which was moved toward the bee's left antenna using a micromanipulator. After touching the antenna, the stimulus was presented for 9 s. Six seconds after stimulus onset, a toothpick either (CS+) soaked in sucrose solution (US) or (CS−) plain was presented to the bee's right antenna. When the bumble bee extended its proboscis in response to the US, it was allowed to lick for 3 s, then the US and CS were removed, and the animal was allowed to rest for 15 s before the next bee was tested (Appendix S3, Figure S2). Each bumble bee was tested in 20 trials (10 rewarded CS+ and 10 unrewarded CS−) in a pseudorandomized order (with a maximum of two identical CS in a row). To standardize conditioning time, we used an intertrial interval (ITI, i.e., time between each trial) of 8 min. Copper plates were cleaned in 99% ethanol, and new filter papers were used for each bee. To prevent the animal from learning the toothpick movement, the toothpick was permanently positioned just below the bee. The bees' responses were recorded using the program Timing-Protocol (Lichtenstein et al., 2015). When the bee showed a PER to the CS (CS+ or CS−), the PER was scored as 1; when the PER was shown only in response to the reward, it was scored as 0; and when the bee did not show any PER, NA was recorded. Bumble bees with five or more NAs were excluded from further analysis. Every tested stimulus was used as CS+ and CS− on different days to prevent any biases.

Stimuli

Stimuli were prepared once a week and then stored at −20°C until further use. All sterols were dissolved 1:1000 in chloroform on ice and stored in leakproof glass vials. Before the conditioning started, 240 filter paper pieces (5 × 5 mm) were freshly prepared each day by applying 5 µL of each stimulus used on the filter paper (120 for CS+ and CS−, respectively.)

The following stimulus combinations were tested: (1) sterol vs. solvent, (2) sterol A vs. sterol B (for details, see

below), (3) a mixture of four sterols in a 10-fold higher concentration than naturally occurring in the bee-collected pollen used in this study (Appendix S1, Table S1 in Appendix S2) vs. solvent, and (4) pure pollen vs. pollen with added sterols.

In total, five sterols were tested: cholesterol, desmosterol, stigmasterol, β-sitosterol, and cholestenone (Sigma-Aldrich, Taufkirchen, Germany; see Appendix S2, Table S1). Their naturally occurring concentrations can vary greatly, from 0.0015 mg/g sitosterol in *Lamium album* up to 2.8955 mg/g β-sitosterol in *Salix caprea* (Vanderplanck et al., 2019; for detailed information see Appendix S2, Table S2). All sterols, except desmosterol, were also tested against each other to examine whether bumble bees were able to differentiate between individual sterols.

Furthermore, a mixture of cholesterol, desmosterol, stigmasterol, and β-sitosterol dissolved in chloroform was tested against the solvent. We used a concentration 10-fold higher than the natural sterol concentration found in the purchased pollen collected by honey bees used in this study (see Appendix S1, Table S1 in Appendix S2). In this experiment, cholestenone was excluded as it was not detected in the used pollen (Appendix S2, Table S1). Since we found that the mixture of pure sterols could be perceived by the bees (see below), we next tested if bees can also discriminate between different sterol concentrations in pollen. For this experiment, the sterol mixture was blended with dry honey-bee-collected pollen that had been ground to a fine powder using a coffee grinder (Bosch, Gerlingen-Schillerhöhe, Germany). The resulting pollen blend was then mixed 1:1 with deionized water to achieve a homogenous pollen paste, and the filter papers were then immersed into the pollen paste. This paste was freshly prepared once at the very beginning of the week and stored at –20°C. Pure pollen paste was then tested against pollen paste enriched with a 10-fold higher sterol concentration.

Feeding experiments

For the feeding experiments, we used 94 queenless microcolonies (46 in the first experimental series, 48 in the second), which show comparable responses compared to queenright colonies in such feeding assays (Tasei and Aupinel, 2008). Each colony was composed of 20 worker bumble bees randomly selected from a single mother colony and kept in small two-chambered wooden boxes (125 × 145 × 105 mm per chamber). Once the bees had been transferred, they were allowed to acclimatize for 72 h, while having access to pure honey-bee-collected pollen and 50% w/w sucrose solution ad libitum. All bees that died within the first 3 days were replaced by new bees. The microcolonies were kept in a climate chamber at 25°C, 60% RH, and 12 h/12 h light/dark.

All microcolonies were randomly assigned to two groups. One group was allowed to rear brood, whereas all laid egg clumps were removed from microcolonies of the second group at least every other day. For each of the two groups, four treatment groups were established and fed with the following diets: (1) pure pollen (control), (2) pollen enriched with a mixture of 0.5× sterol concentration (SC), (3) pollen enriched with 5× SC, and (4) pollen enriched with 10× SC. The sterol mixture comprised cholesterol, stigmasterol, and β-sitosterol (note that we omitted desmosterol due to its high cost). The pollen was dry-ground into a fine powder, mixed with sterols, and ground again to achieve a homogeneous sterol pollen mixture, and stored at –20°C until further use. Before use, the pollen was mixed 1:1 with deionized water to achieve a homogeneous pollen paste and stored in the freezer.

The feeding experiment ran for 21 days. Colonies had ad libitum access to sugar water (50% w/w) and were provided with 1–2 g of fresh pollen paste (the amount was adjusted to colony development during the experiment to ensure that the pollen was never depleted) in a small petri dish per day. The dishes were weighed daily to determine the pollen consumption of each microcolony. To correct for evaporation, a dish with each diet was placed outside the colonies in an empty box and weighed to quantify daily water loss. This mass loss was then subtracted from the mass loss of the pollen paste within the colonies. Each day, the amount of new egg clumps, larvae, and pupae and number of bees that died were recorded. Dead bees were not replaced after the first three acclimation days. After the experiment had been terminated, each nest was dissected, and final number of egg clumps, larvae and pupae were counted.

Statistical analyses

All statistical analyses were performed using R version 4.1.2 (R Core Team, 2021).

PER experiments

Each stimulus was tested as a CS+ and a CS− on different days to prevent reward bias (e.g., on day 1 cholesterol was the rewarded stimulus and stigmasterol the unrewarded one, and on day 2 stigmasterol was the rewarded stimulus and cholesterol the unrewarded one). Since we found no reward bias for any stimulus pair, we pooled the data (e.g., the results for cholesterol CS+ and stigmasterol CS− were pooled together with the results for cholesterol CS− and stigmasterol CS+). We further tested for any variance between experimental series 1 and 2 by means of a *U*-test. This was necessary, as three of the sterol combinations (cholestenone vs. cholesterol, cholestenone vs. stigmasterol, cholesterol vs. stigmasterol) were tested in both experimental series. No significant differences were found between the experimental series, and we therefore pooled the data. Finally, we compared the PER responses between CS+ and CS− for each PER experiment by ranking each individual according to its number of correct responses (0–10). These ranks were compared between the rewarded and unrewarded stimulus by a paired *U*-test.

Feeding experiments

All parametric response variables were tested for normal distribution using Shapiro–Wilk's test and for homogeneity of variances using Levene's test included in the R package car (Fox and Weisberg, 2019). We additionally tested whether the data had a significant variance between the two experimental series by means of a *U*-test (since data were not normally distributed). Where no significant differences were found, data were pooled. Otherwise, data were analyzed separately.

To assess whether pollen consumption of *Bombus terrestris* workers (per individual and per day) was affected by different concentrations of sterols in pollen or presence/absence of brood over the time of the experiment (instead of mean consumption for the total experiment; see Appendix S3, Figure S3), we performed generalized additive

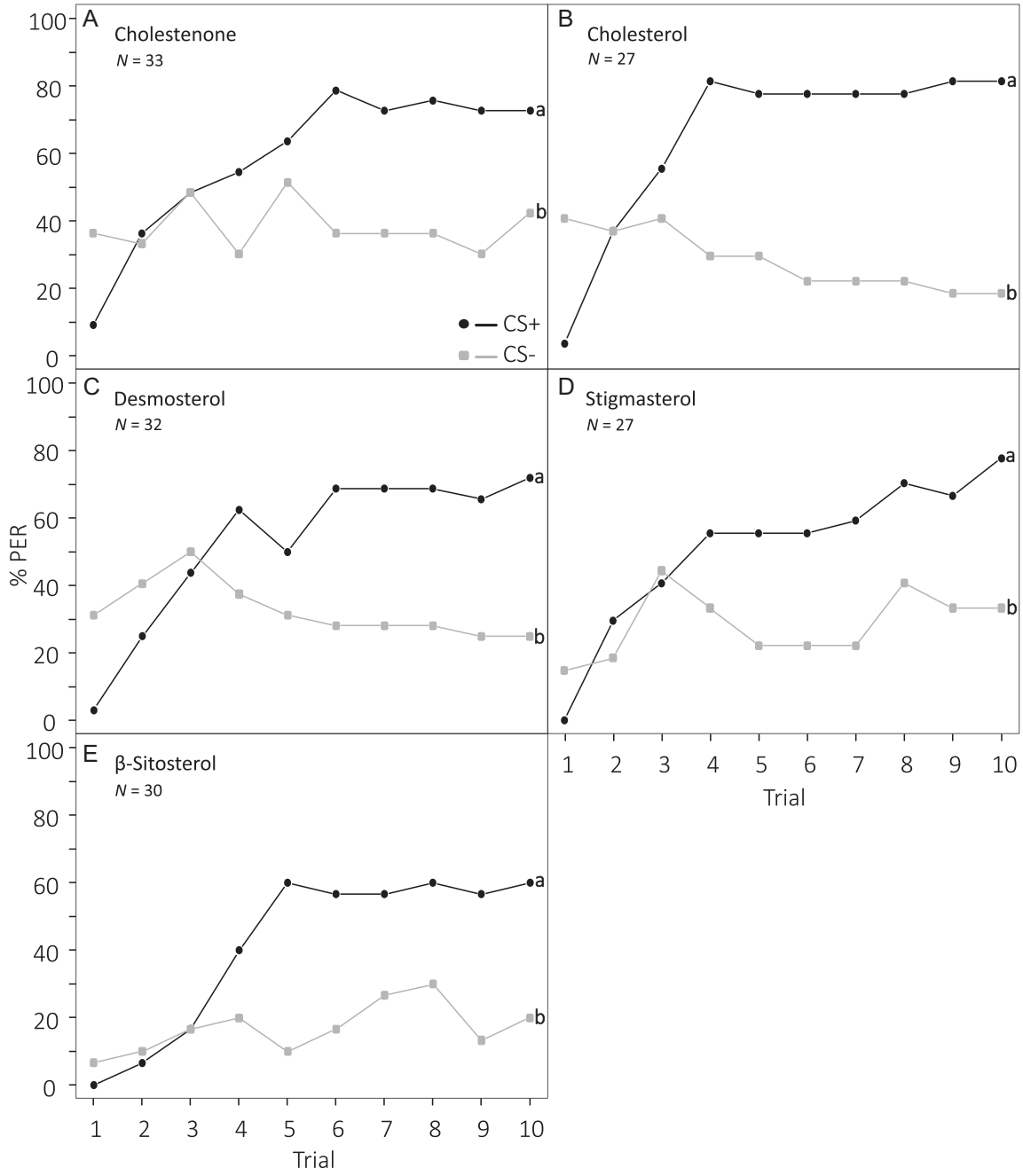

FIGURE 1 Percentage of *Bombus terrestris* workers ($N = 179$) that responded with a proboscis extension response (PER) to one out of five sterols when individually tested against the solvent control (chloroform). (A) Cholestenone ($N = 33$, $U = 6$, $P = 0.0001$), (B) cholesterol ($N = 27$, $U = 5$, $P = 0.0001$), (C) desmosterol ($N = 32$, $U = 30.5$, $P = 0.0007$), (D) stigmasterol ($N = 27$, $U = 15$, $P = 0.0005$), and (E) β-sitosterol ($N = 30$, $U = 21$, $P = 0.0004$). CS+ (black, circles) represents the rewarded stimulus and CS– (grey, squares) the unrewarded stimulus. Both sterol and solvent were tested as CS+ and CS–. Because there was no significant difference between which stimulus was used as CS+ or CS– for any conditioning trial, we combined the data. Data across trials were pooled for the *U*-test. Significant differences are marked with different letters at the right side of the curves ($P < 0.05$).

mixed-effect models (GAMM), with microcolony included as random factors. Since there was a high daily (nonlinear) variance in the consumption, an additive model approach, smoothening the data over the days, was chosen. If needed, data were log-transformed to achieve normal distribution and homogeneity of variance. Data of both experimental periods were pooled.

To evaluate the effect of the sterol diets on the overall reproductive success of microcolonies, i.e., the number of egg clumps, larvae, and pupae on the last day, we used a generalized linear mixed effect model (GLMM; R package lme4, Bates et al., 2015) as described by Ruedenauer et al. (2020). We first tested whether mother colony should be included as a random effect in this model by comparing a GLMM to a generalized

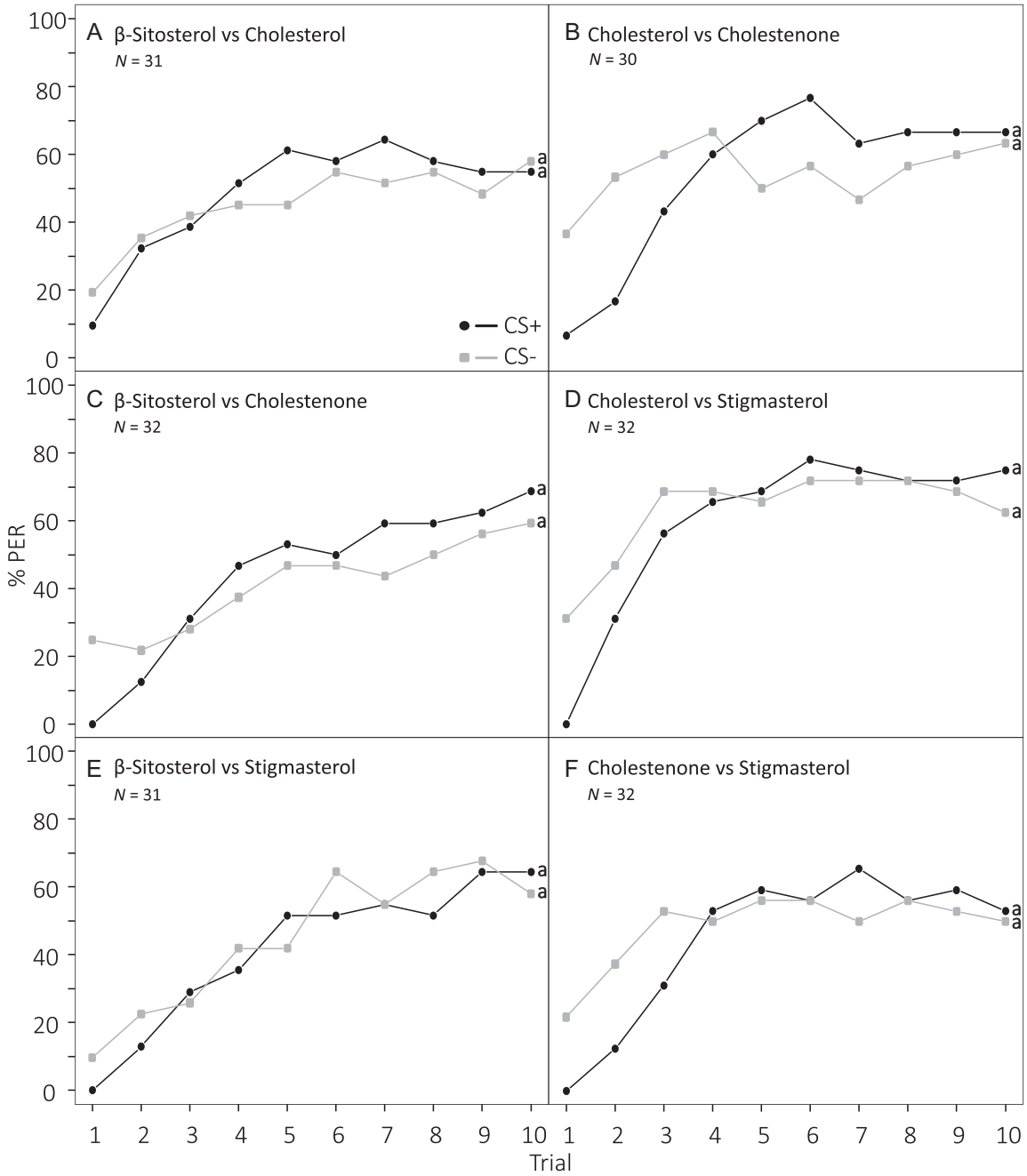

FIGURE 2 Percentage of *Bombus terrestris* individuals ($N = 188$) that responded with a proboscis extension response (PER) in a differential conditioning experiment: (A) β-sitosterol vs. cholesterol ($N = 31$, $U = 44.5$, $P = 0.232$), (B) cholesterol vs. cholestenone ($N = 30$, $U = 135.5$, $P = 0.496$), (C) β-sitosterol vs. cholestenone ($N = 32$, $U = 123.5$, $P = 0.299$), (D) cholesterol vs. stigmasterol ($N = 32$, $U = 188.5$, $P = 0.126$), (E) β-sitosterol vs stigmasterol ($N = 31$, $U = 136$, $P = 0.253$), (F) cholestenone vs stigmasterol ($N = 32$, $U = 102$, $P = 0.082$). CS+ (black, circles) represents the rewarded stimuli and CS− (grey, squares) the unrewarded stimulus. Both sterols were tested as CS+ and CS−. Because there was no significant difference between which stimulus was used as CS+ or CS− for any conditioning trial, we combined the data. Data across trials were pooled for the *U*-test. The same letters at the right side of the curves indicate no significant differences.

linear model (GLM, lme4 package) without random effect (both with Gaussian distribution) via a likelihood ratio test for model comparison. This comparison revealed that the random effect explained a significant proportion of the variance. We therefore performed a GLMM including mother colony as a random factor. Data of both experimental periods were pooled. To examine differences in longevity of bumble bees exposed to different diets, we used Kaplan–Meyer survival statistics by comparing median survival times and tested every diet against each other (packages survival [Therneau and Grambsch, 2013] and KMsurvpackage [Klein and Moeschberger, 2006]). Survival curves differed significantly between the two experimental periods and were therefore analyzed separately. Due to multiple testing, α-levels were adjusted using the Bonferroni correction.

RESULTS

PER experiments

Bumble bees were able to discriminate any tested sterol from the solvent control (cholesterol vs. control, $U = 5$, $P = 0.0001$; cholestenone vs. control, $U = 6$, $P = 0.0001$; desmosterol vs. control, $U = 30.5$, $P = 0.0007$; stigmasterol vs. control, $U = 15$, $P = 0.0005$ and β-sitosterol vs. control, $U = 21$, $P = 0.0004$; Figure 1). However, they were not able to differentiate between different sterols (β-sitosterol vs. cholesterol, $U = 44.5$, $P = 0.232$; cholesterol vs. cholestenone, $U = 135.5$, $P = 0.496$; β-sitosterol vs. cholestenone, $U = 123.5$, $P = 0.299$; cholesterol vs. stigmasterol, $U = 188.5$, $P = 0.126$; β-sitosterol vs. stigmasterol, $U = 136$, $P = 0.253$; cholestenone vs. stigmasterol, $U = 102$, $P = 0.082$; Figure 2).

Bees also were able to discriminate between the mixture of four sterols and the solvent control ($U = 0$, $P = 0.0005$; Figure 3A). However, they were not able to discriminate between pollen enriched with the 10-fold higher sterol mixture and pure pollen ($U = 71$, $P = 0.541$; Figure 3B).

Feeding experiments

The different pollen diets (pure pollen or pollen with low, medium, or high sterol content) did not affect individual consumption over time either for microcolonies with brood ($F_{1,3} = 0.746$, $P = 0.525$) or for microcolonies without brood ($F_{1,3} = 1.1$, $P = 0.348$; Figure 4). The same was true for the average amount of pollen consumed per individual and day (first experimental series: $F_{1,3} = 0.557$, $P = 0.644$ and second: $F_{1,3} = 1.229$, $P = 0.298$; Appendix S3: Figure S3). In microcolonies with brood, the average pollen consumption per individual and day increased for all colonies independent of diet from an average of 13.1 mg (±9.6) on the first day to 63.7 (±33.3) mg on day 19. In broodless colonies, average pollen consumption per individual and day remained constant at around 10.8 mg (±9.0) per individual. Reproduction was also not affected by the different diets. No significant differences were found in the number of egg clumps ($F_{1,3} = 2.024$, $P = 0.125$), larvae ($F_{1,3} = 1.845$, $P = 0.182$) or pupae ($F_{1,3} = 2.025$, $P = 0.154$; Figure 5). Similarly, there was no effect of diet on the day of the first occurrence of egg clumps ($F_{1,3} = 0.952$, $P = 0.415$), larvae ($F_{1,3} = 0.477$, $P = 0.698$), or pupae ($F_{1,3} = 1.453$, $P = 0.226$; Appendix S3: Figure S4). Notably, worker longevity differed significantly between the two experimental series ($P < 0.005$), but not between diets

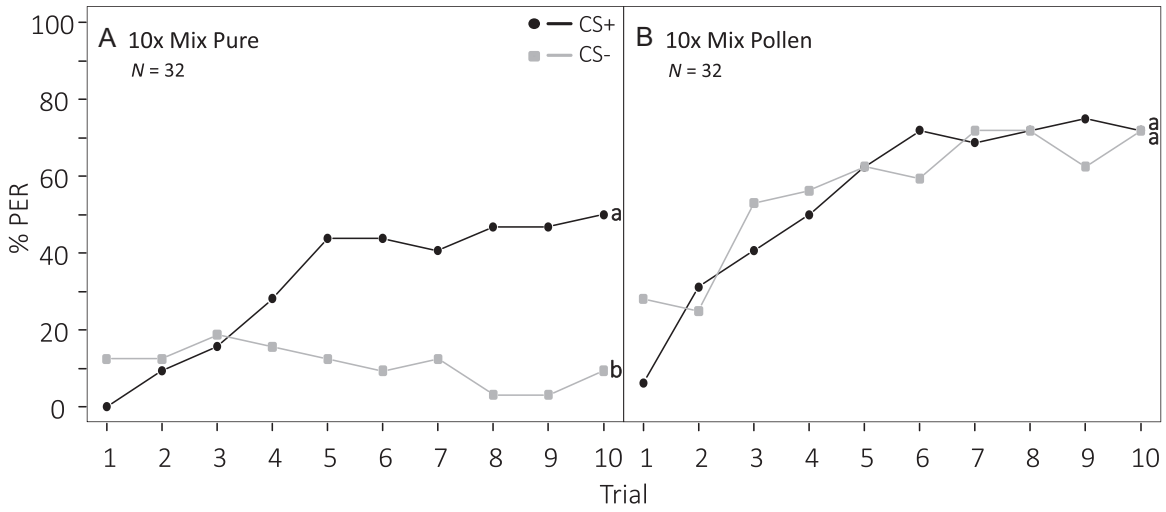

FIGURE 3 Percentage of *Bombus terrestris* individuals ($N = 64$) that responded with a proboscis extension response (PER) when tested with a sterol blend. (A) 10× sterol mixture vs. solvent ($N = 32$, $U = 0$, $P = 0.0005$), (B) 10× sterol mixture in pollen vs. pure pollen ($N = 32$, $U = 71$, $P = 0.541$). CS+ (black, circles) represents the rewarded stimuli and CS– (grey, squares) the unrewarded stimulus. Both sterol, sterol–pollen mixture, and solvent were tested as CS+ and CS–. Because there was no significant difference between which stimulus was used as CS+ or CS– for any conditioning trial, we combined the data. Data across trials were pooled for the *U*-test. Significant differences are marked with different letters at the right side of the curves ($P < 0.05$).

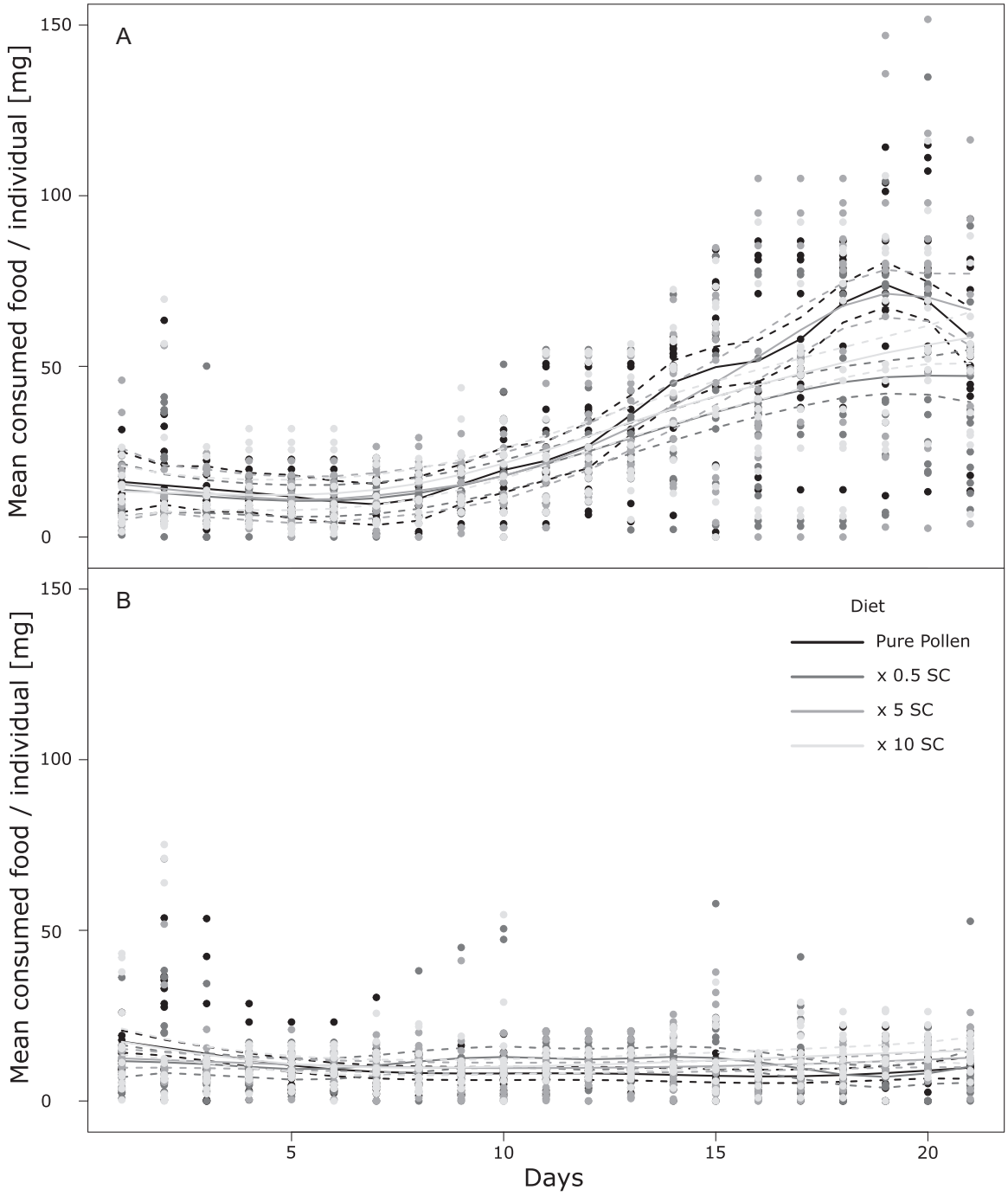

FIGURE 4 Mean amount of consumed food per individual and day of *Bombus terrestris* microcolonies fed pollen diets differing in sterol content (SC; pure pollen, ×0.5, ×5, and ×10 the natural concentration) ($N = 94$). Colonies were either (A) allowed to rear brood or (B) had their brood removed every other day. Each dot represents the average pollen consumption per individual and day for one microcolony. Continuous lines represent smoothers calculated by generalized additive mixed effect models (GAMMs) with dashed lines as upper and lower 95% confidence intervals. The different sterol concentrations did not affect the amount of food consumed per individual in (A) ($F_{1,3} = 0.746$, $P = 0.525$) or (B) ($F_{1,3} = 1.1$, $P = 0.348$). This graph represents the pooled data from both experimental periods.

(experimental series 1, $P = 0.32$ and experimental series 2, $P = 0.31$). Further, only in the first experimental series was a tendency toward reduced worker longevity of colonies without brood compared to colonies with brood (experimental series 1, $P = 0.053$; experimental series 2, $P = 0.7$; Figure 6).

DISCUSSION

Our results show that *B. terrestris* workers can perceive various sterols, but cannot differentiate them. Interestingly, when the same sterols were added to pollen, the bees were unable to differentiate between the sterol-enriched pollen

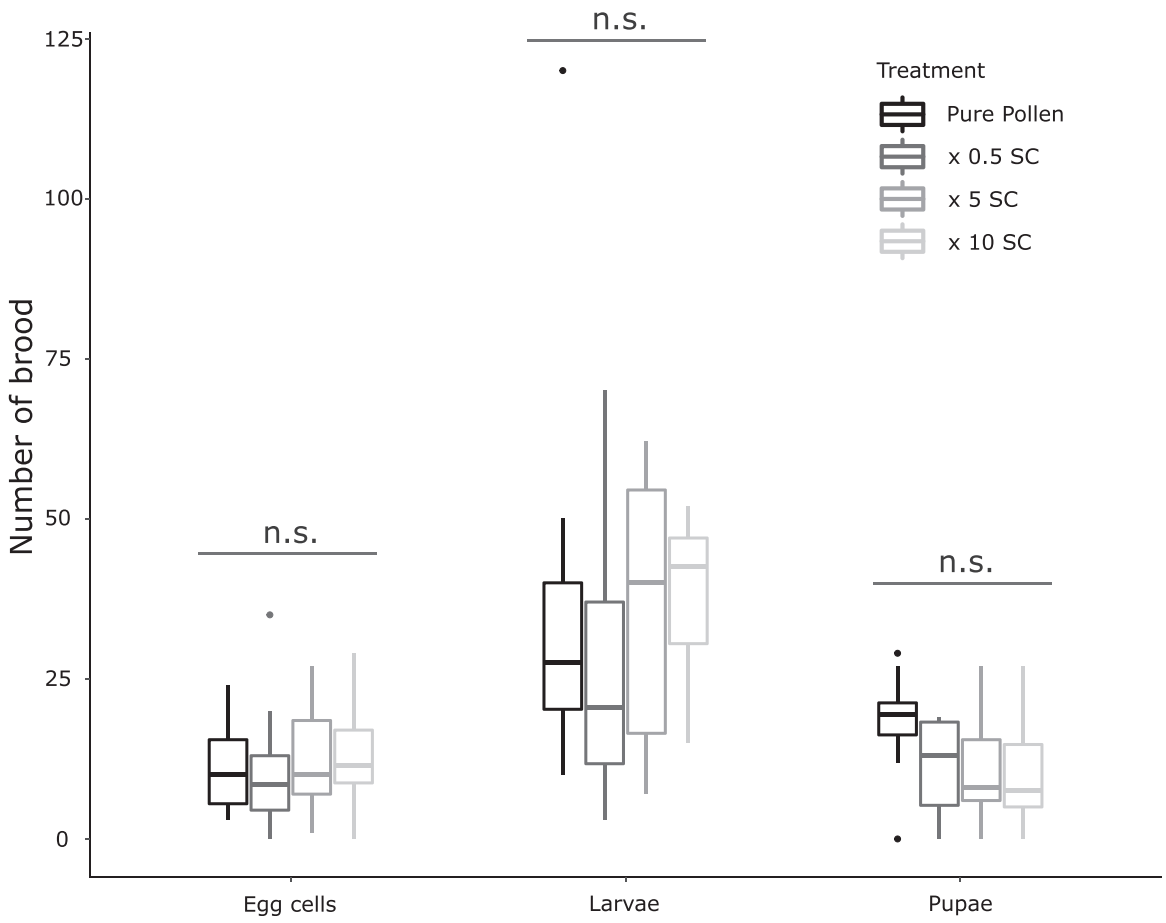

FIGURE 5 Number of egg clumps, larvae, and pupae of *Bombus terrestris* microcolonies ($N = 94$) fed with pollen diets differing in sterol content (SC; i.e., pure pollen, ×0.5, ×5, and ×10 times the natural concentration) on the last day of the feeding experiments. Pollen diet had no effect on the number of egg cells ($F_{1,3} = 2.0238$, $P = 0.1247$), larvae ($F_{1,3} = 1.84485$, $P = 0.1824$), or pupae ($F_{1,3} = 2.02522$, $P = 0.1536$). n.s., not significant.

and pure pollen. Moreover, differences in the content of those sterols used in our experiment did also not affect colony performance in *B. terrestris* microcolonies. These findings at least partly contradict our previous assumption that strong variations in sterol content should affect bee health due to their known importance. However, because we only used relatively high pollen sterol concentrations in our experiment, we cannot exclude that bees can detect sterols in pollen at concentrations below a specific threshold and would avoid pollen with, e.g., very low sterol concentrations. Future experiments should therefore additionally compare artificial pollen surrogate with no or very low sterol content (e.g., based on casein) and pollen with natural sterol concentrations to determine if the perception of pollen sterols is concentration dependent. Studies with other insect species showed that *Drosophila* mutants with the cholesterol regulating receptor knocked out consumed excess amounts of cholesterol, which led to a higher mortality (Horner et al., 2009). On the other hand, sterol deficits impacted on growth in *Drosophila* larvae (Carvalho et al., 2010). Similarly, sterol amounts above a threshold level decreased fitness in various caterpillar species (Jing et al., 2012, 2014). It is therefore surprising that *B. terrestris* cannot perceive the tested sterol concentration differences in pollen by means of its antennae.

Interestingly, the bees could detect sterols when they were presented in isolation (although not discriminate between them), which strongly indicates that *B. terrestris* does have the basic receptor(s) to detect as well as the neuronal means to perceive sterols in general. Like fatty acids, sterols belong to the highly diverse lipids. Lipids are lipophilic (soluble in organic solvents) and contain hydrocarbon chains or rings as their principal chemical structure. In contrast to their inability to discriminate sterols, bumble bees can perceive and differentiate between fatty acids (Ruedenauer et al., 2020). In general, receptors for macronutrients such as sugars, amino acids, and fatty acids are known from mammals (Hoon et al., 1999) and some insects (Clyne et al., 2000; Hallem et al., 2006). For example, Brown et al. (2021) identified gustatory receptors for fatty acids in *Drosophila*, which are expressed in gustatory receptor neurons (GRNs) located in their tarsi, proboscis, pharynx and wings and enable *Drosophila* to discriminate between fatty acids differing in carbon chain length. However, sterols differ in their chemical structure from fatty acids in that they comprise several hydrocarbon

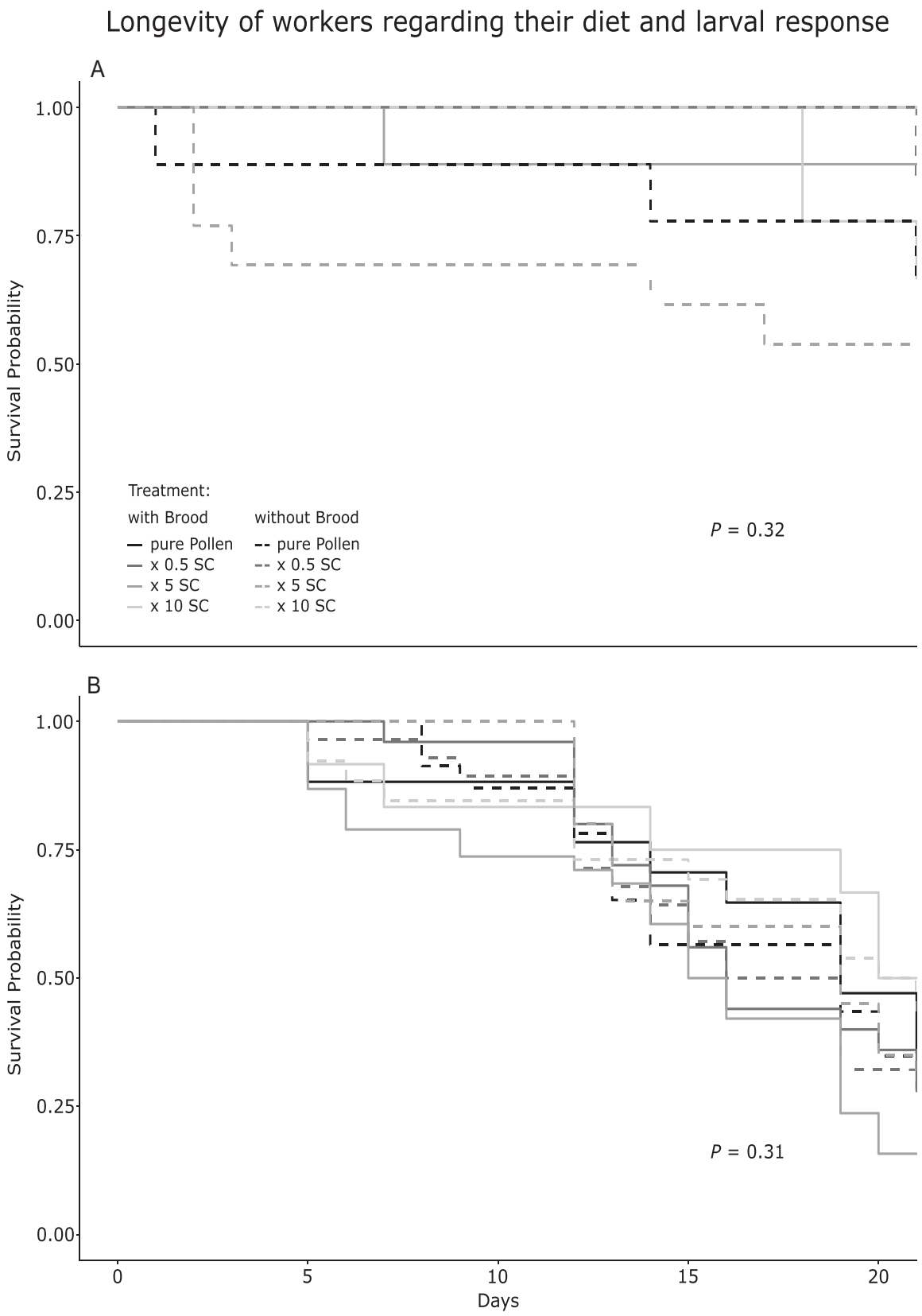

FIGURE 6 Survival of *Bombus terrestris* workers in microcolonies fed pollen diets differing in sterol content (SC) (i.e., pure pollen or pollen enriched with ×0.5, ×5, or ×10 the natural concentrations of a sterol mixture) and either allowed to raise brood (with brood) or not (without brood). The experiments were conducted in two different experimental series (A and B), which were analyzed separately. There was no difference in survival probability of individuals fed different diets in experimental series A ($P = 0.32$) and B ($P = 0.31$). Note that in experimental series A, the treatment groups pure pollen, ×5 SC and ×10 SC (all with brood) no worker died (100% survival after 3 weeks).

rings instead of chains. They are also often amphiphilic (hydro- and lipophile parts combined), and it is thus likely that sterols require a different repertoire of gustatory receptors than fatty acids.

Even though *B. terrestris* workers principally were able to detect sterols, it appears that the bees do not pay specific attention to the sterol content (at least for the sterols we tested) when presented in pollen, potentially because pollen is a highly diverse mixture of many different compounds. A similar phenomenon was found for amino acid perception in *B. terrestris*: while bumble bees could learn and thus perceive concentration differences for some amino acids when presented in isolation, they were not able to differentiate between them (Ruedenauer et al., 2019a) and also "ignored" them when they were mixed with pollen (Ruedenauer et al., 2020). Notably, pollen represents a complex mixture of many different nutrients and compound groups, such as various macro- and micronutrients and plant secondary metabolites, which differ in their effect on bee health and fitness (Roulston and Cane, 2000; Campos et al., 2008; Filipiak et al., 2017; Palmer-Young et al., 2019). Assessing the full spectrum of pollen compounds might be very time- and energy-consuming for a bee, as it likely requires a suite of receptors as well as perceptional and behavioral responses. Therefore, bees may focus on the most important and/or most detrimental compounds in pollen, and in fact, *B. terrestris* workers appear to focus their perception on pollen fatty acids, while ignoring other macronutrients like amino acids (Ruedenauer et al., 2020). Moreover, changes in the concentrations of fatty acids in pollen, as naturally occurring in plants, strongly affected the bees' survival, reproductive fitness, and pollen intake (Ruedenauer et al., 2020). By contrast, our feeding experiments suggest that the consumption of pollen enriched with sterols in concentrations as naturally occurring in plants (Rasmont et al., 2005; Vanderplanck et al., 2014, 2019; Somme et al., 2015; Villette et al., 2015) does not have any obvious detrimental effect on bumble bee survival and reproductive fitness. Moreover, sterol concentrations slightly above naturally occurring ones, as used in our feeding experiments (Vanderplanck et al., 2014, 2019; Somme et al., 2015), also did not affect any measured fitness parameters, suggesting that *B. terrestris* does not need to prioritize sterol perception and can easily tolerate variations in those concentrations as tested in our experiment. An alternative explanation for our findings is that bees require only a very small amount of phytosterols, and as soon as this threshold is met, "ignore" the pollen sterol content. If so, the bees might detect the presence of sterols but not assess their absolute concentrations or ratios. To test this hypothesis, artificial pollen surrogates, as mentioned above, should be used in future studies. Overall, our results also indicate that bees may have less-specific requirements for pollen sterols as previously suggested (Vanderplanck et al., 2019, 2020), at least for those sterols tested in our experiments.

Notably, we only performed a forced feeding assay, where the bees are offered just one specific diet, instead of a two-choice feeding assay, where the bees can choose between two diets. We can therefore not rule out that post-ingestive perception mechanisms might influence pollen choice based on its sterol content. Another limitation of our study is the restricted spectrum of sterols used in the experiments. Notably, 24-methylenecholesterol and campesterol were found to be the most abundant phytosterols in pollen (Vanderplanck et al., 2014, 2019, 2020; Somme et al., 2015), but were not tested in our study. Although we expect cholesterol to be essential for bumble bees (like it is for most of the insects, see Svoboda et al. 1978), it is possible that bumble bees rely on different sterols from those we tested here. Honey bees, for example, depend on campesterol or sitosterol instead of cholesterol (Svoboda et al., 1983, 1994; Feldlaufer et al., 1985; Jing and Behmer, 2020; Zu et al., 2021). It is also likely that sterols differently affect distinct bee species, as has been shown for other nutrients by Barraud et al. (2022). The authors showed that *Apis mellifera* performed worse than *Bombus terrestris*, *Osmia cornuta* and *O. bicornis* on a protein-rich diet (36.9%). They also found a decrease in body mass (22–26%) in *O. cornuta* and *O. bicornis* that fed on a diet with low amounts of essential amino acids, but not in *A. mellifera*.

CONCLUSIONS

Our findings suggest that variations in sterol concentration (i.e., cholesterol, cholestenone, desmosterol, stigmasterol, β-sitosterol) within the range tested in our study are "ignored" by *B. terrestris* workers and do not affect their colony performance. Because we used naturally occurring as well as slightly higher concentrations found in pollen, our results further indicate that *B. terrestris* workers may not need to pay specific attention to pollen sterol content, since the range of sterol concentrations usually found in pollen is not detrimental to their survival and reproductive fitness. Future studies need to address whether the bees can detect a lower limit in pollen sterol concentrations to avoid malnutrition as a consequence of a sterol deficit and whether some sterols are more important than others.

AUTHOR CONTRIBUTIONS

J.S., F.A.R., and S.D.L. conceived the experiment; C.A.N. and M.C.S. performed the experiments; F.A.R., C.A.N., and M.C.S. analyzed the data. All authors drafted the manuscript, commented on the draft, and agreed to the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare they have no competing interests.

DATA AVAILABILITY STATEMENT

All relevant data are available at OSF at: https://doi.org/10.17605/OSF.IO/QH839.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

APPENDIX S1. Protocol for pollen analysis.

APPENDIX S2. Sterols tested and sterol concentrations in pollen.

APPENDIX S3. Experimental setup; mean consumption of pollen per bee individual per day; cumulative proportion of microcolonies that produced eggs, larvae, or pupae.

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