UNIVERSITY OF CALIFORNIA, SAN DIEGO

Effects of chronic exposure to flupyradifurone and low-quality sucrose on honey bee flight and mortality

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in

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by

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The Thesis of Linda Tong is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

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ABSTRACT OF THE THESIS

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Insecticides pose a major health concern for honey bee colonies, particularly those involved in crop pollination. Flupyradifurone (commercially known as Sivanto) is a new butenolide insecticide that has been marketed as bee-safe, and is especially used in cases where pest insects have evolved resistance to neonicotinoid pesticides. In addition to insecticides, honey bee health is also affected by decreases in food source diversity and quality, as can happen in

agricultural monocultures. We tested the effects of flupyradifurone exposure and food quality (sucrose concentration) on European honey bee flight performance and mortality. Flight performance is critical to colony health because flight is required for worker bees to forage for food and to return safely to the nest. At our field site, bees flew to collect pollen and nectar throughout the year; we therefore analyzed FLU effects in the winter and the summer. We found that a high-quality sucrose diet increased survival in foragers ($p<0.0001$) over the winter and summer. Exposure to FLU caused bees to consume less of the high-quality sucrose diet in summer ($p<0.05$), and bees that consumed low-quality sucrose ingested more FLU than bees that consumed high-quality sucrose ($p<0.0001$). Control foragers flew longer ($p=0.029$) and farther ($p=0.046$) in the summer than in the winter. However, FLU consumption eliminated these seasonal differences in flight duration and distance. FLU also significantly decreased the percentage of successful flights when bees consumed low-quality 33% sucrose ($p=0.0013$). Higher quality sucrose significantly increased the temperature difference between thoracic temperature before flight—a measure of wing muscle temperature—and ambient air temperature ($\Delta T_{\text{before flight}}$). Thoracic temperature ($T_{\text{before flight}}$) was positively, though weakly, correlated with flight velocity ($p\leq0.027$). Because agricultural insecticide exposure and poor nectar quality can co-occur, we suggest that further studies are necessary to evaluate the safety of FLU for honey bees.

INTRODUCTION

The European honey bee, *Apis mellifera*, provides crucial ecosystem services through pollination of native plants and crops worldwide (Potts *et al.* 2010). Insect pollination is needed for 75% of crops, and honey bees are the predominant pollinator (Potts *et al.* 2010). However, managed honey bee populations have decreased globally in the last several decades. During the winter of 2013-2014 almost 45% of colonies were lost in the U.S. compared to an average of 5-10% loss before the 1980s (Lee *et al.* 2015). From 2014-2015, total annual colony losses amounted to over 40% (Seitz *et al.* 2016). Large annual losses of managed honey bees are problematic given their role in crop and even native plant pollination, and such losses increase the costs of maintaining healthy bee stocks sufficient for agricultural pollination (Seitz *et al.* 2016). Recent declines in honey bee colonies may therefore have substantial impacts on the primary productivity of crop production and perhaps even native ecosystems (Klein *et al.* 2007).

Factors contributing to recent honey bee losses include infection by pathogens and parasites (Toplak *et al.* 2013), exposure to agricultural chemicals (Sanchez-Bayo 2014; Henry *et al*. 2015), environmental variation leading to malnutrition (Klein *et al.* 2007), and synergistic effects between these factors (Potts *et al.* 2010). Insecticides have received attention because although they target pest insects, they also affect pollinating insects. Honey bees use pesticide-treated crops as a food source, and chemical residues can persist within the honey bee (Henry *et al.* 2012) and in soil even after pesticide use has ceased (Sanchez-Bayo 2014). For example, neonicotinoid insecticides effectively target sucking pests such as aphids (Jeschke & Nauen 2008), but are also harmful to insect pollinators such as honey bees (Henry *et al.* 2012). These insecticides may be contributing to annual honey bee losses (van der Sluijs *et al.* 2013).

Neonicotinoids are agonists of nicotinic acetylcholine receptors (nAChR) and can lead to paralysis and death in insects (Matsuda *et al.* 2001). In honey bees a common neonicotinoid,

imidacloprid, increases brain cell death (Wu *et al.* 2015), reduces associative olfactory learning (Sanchez-Bayo 2014; Zhang & Nieh 2015), and decreases efficiency of foraging behavior (Yang *et al.* 2008). Exposure to Thiamethoxam (TMX), another common neonicotinoid, can cause high mortality in honey bees by impairing homing (Henry *et al.* 2012). In experiments conducted with forager honey bees marked with radio-frequency identification (RFID) tags, bees exposed to TMX were unable to find the way back to their home sites, thereby decreasing colony fitness (Henry *et al.* 2012). Recent studies have also shown that TMX may significantly impair the physical ability of bees to fly (Tosi *et al.* 2017). Flight duration, distance, average velocity and maximum velocity significantly decreased after chronic exposure to TMX (Tosi *et al.* 2017).

In response to these concerns about neonicotinoids and to growing pest resistance to the neonicotinoids, a new generation of pesticides has been developed. Flupyradifurone (FLU), commercially known as Sivanto^{TM}, is a butenolide insecticide that is chemically similar to neonicotinoids (Bayer CropScience 2013). FLU is based upon a natural product, stemofoline, that binds to insect nicotinic acetylcholine receptors and is distinguished from the neonicotinoids by a butenolide scaffold in its head group *(Nauen et al. 2015).* FLU also binds to nAChR, but is reversible and works at a different site from neonicotinoids (Campbell *et al.* 2016). Therefore, how FLU binds and is metabolized is distinct from neonicotinoids. FLU is effective against sucking pests that are resistant to neonicotinoids, such as imidacloprid, and FLU is reportedly safer for non-target organisms, particularly honey bees (Nauen *et al.* 2015). Common agricultural applications include use on citrus, cocoa, cotton, grapes, hops, pome fruits, potatoes, soybeans, and ornamental plants (Nauen *et al.* 2015).

Published research testing the effects of FLU on honey bees is limited. However, multiple studies have been submitted to the EPA as part of the permit process (EPA 2014). These honey bee FLU experiments have investigated exposure based on foliar application, soil treatment, and seed treatment residues (EPA 2014 DP: D415164; PC: 122304). Tests included

oral and contact-based acute exposure (EPA 2014: MRID 48843722 and 48844514). Semi-field (MRIDs 48844531 to 48844536) and full field (MRIDs 48844516 and 48844517) studies were conducted along with colony feeding experiments in which FLU was mixed with a diet of nectar and pollen (MRID 48843771). In the field and colony feeding studies, there were no significant reported adverse effects on honey bee behavior, survival, colony health or brood development.

Field studies have suggested that there are no long-term adverse effects of FLU on actively foraging honey bees, up to 200 grams of active ingredient per hectare (g a.i./ha) (Bayer CropScience 2013). However, only two long-term field studies have been completed, to date, and recommended field applications range from 100 to 400 g a.i./ha (Bayer CropScience 2013). Exceeding field-realistic levels of FLU can be toxic. Acute oral exposure to FLU, through consumption of residues in pollen and nectar, is highly toxic to honey bees (Sivanto 200SL: LD50 3.2 µg a.i./honey bee; BYI 02960 technical grade: LD50 1.2 µg a.i./honey bee) (Bayer CropScience 2013; Campbell et al. 2016). In semi-field studies, there were only temporary increases in mortality in treated versus untreated colonies. Full field studies did not show any detectable adverse effects on treated honey bees (Bayer CropScience 2013). Based upon these studies, FLU has been permitted for use on a wide variety of crops in the USA and Europe (EPA 2014 DP: D415164/PC: 122304, EFSA 2014).

However, no studies have yet examined the effects of FLU on the ability of bees to fly. We therefore used a standard assay of bee flight ability: bees flying in flight mills (Well *et al*. 2016; Attisano *et al*. 2015). We focused on flight behavior because locomotion and flight are required for colony food intake, and essential for colony fitness and pollination services.

Flight muscle temperature is an important factor in efficient honey bee flight (Heinrich 1974). Honey bees can regulate their thoracic temperatures above ambient air temperatures during flight, and flight duration; distance; and speed are related to flight muscle temperatures (Bernd 1979). This thermoregulation for flight requires energy, and the energy is provided by

nectar sugar content (Gould & Gould 1988). Therefore, flight speed may be positively correlated with nectar sugar concentration (Gmeinbauer & Crailsheim 1993). The quality of available nectar can fluctuate greatly, as natural nectar sugar concentrations can vary from 0.2 M to 2.0 M (Gould & Gould 1988). In agricultural monocultures, reductions in floral diversity may limit the quality of nectar that bees can access, and consequently limit the energy available for flight (Klein *et al.* 2007).

Seasonality can also influence bee flight (Park & Nieh 2017). Honey bees can adapt to seasonal changes and food scarcity by modifying their foraging range (Schneider & McNally 1993) and recruitment strategies (Park & Nieh 2017). Winter bees have a higher mortality rate than summer bees and are affected differently by stressors such as neonicotinoids (Decourtye *et al.* 2003). We therefore also considered season in our study. We split our study period into two seasons, winter (September to February) and summer (March to August) to reflect the cool and wet dormant season, and warm and dry growth season, respectively (Park & Nieh 2017).

We examined the effects of FLU, food quality (high vs. low quality sucrose diets), flight muscle temperature (measured as thoracic surface temperature), and season on flight ability. We hypothesized that sublethal doses of FLU and low-quality sucrose diet would decrease honey bee survival, decrease thermoregulation and negatively affect bee flight, thereby impairing bee foraging activity and colony health.

MATERIALS AND METHODS

Study site and colonies

This study was conducted from April 2016 to April 2017 at the University of California, San Diego. Foragers were collected from six healthy honey bee colonies (*A. mellifera ligustica* Spinola, 1806, 10 frames per colony) housed in an apiary at the UC San Diego Biology Field Station. Standard inspection and treatment techniques (Higes *et al.* 2011; Dietemann *et al.* 2013) were used: colonies were healthy and not infected by *Varroa* mites or *Nosema*.

Seasonality

Our study period was split into two seasons: March to August was categorized as summer, the growth season; September to February was winter, the relatively dormant season (Park & Nieh 2017). At our research site, bees would forage and recruit (waggle dance) for natural pollen sources throughout the year, though with lower activity during December, January and February (Park & Nieh 2017). FLU is largely used, to date, in agricultural crops: citrus, cocoa, cotton, grapes, hops, pome fruits, potatoes, and soybeans (Nauen *et al.* 2015). However, it is also used in ornamental plants (Nauen *et al.* 2015) that can flower throughout the year. Flowering times for FLU-treated crops also occur in both of our seasons.

Collection and feeding

We collected returning pollen foragers, identified as returning bees carrying pollen loads, at the entrances of their hives. We used pollen foragers because they are easily identifiable as foragers and, as such, have been used in multiple other studies (Henry *et al.* 2015; Tosi *et al.* 2017). Bees were transferred into separate cages (10 bees per cage), each with a different treatment: 50% w/w pure sucrose solution, 50% sucrose solution with 4 ppm FLU, 33% w/w pure

sucrose solution, and 33% sucrose solution with 4 ppm FLU. Sucrose content of 50% is consistent with natural nectar sucrose concentrations (Gould & Gould 1988), and field-realistic concentrations of FLU are at 4 ppm (Rexer 2012a, 2012b). Each cage was provided with 1.0 ml of treatment solution that was weighed each 24 h period (see below).

Pesticide concentrations

We used analytical grade flupyradifurone (FLU) (Sigma Aldrich, CAS# 951659-40-8, catalog# 37050-100MG) to create our pesticide treatment solutions. Solutions were prepared weekly in 50 mL tubes using double-distilled water, and the tubes were wrapped in aluminum foil to prevent light degradation of the FLU (Bonmatin *et al.* 2015). Solutions were stored at 4° C. A concentration of 4.3 ppm (4300 µg/kg) and 4.1 ppm (4108 µg/kg) of FLU was found in the honey stomach of foragers collecting nectar from oilseed rape fields treated with recommended FLU concentration in France and Northern Germany (Rexer 2012a; Rexer 2012b). Thus, in our study we exposed bees to a field-realistic concentration of FLU: 4 ppm (4 µg/kg).

Chronic exposure experiment

To test the effects of field-realistic FLU exposure and sucrose diet concentration on honey bee flight ability and mortality, pollen foragers were fed *ad libitum* 50% sucrose with FLU (4 ppm) or without FLU, or 33% sucrose with FLU (4 ppm) or without FLU. Foragers were incubated at 30° C and 50-80% RH for 3 days. Bees in each cage were chronically exposed by being fed, *ad libitum* with a syringe filled with sucrose solution to simulate pesticide exposure over multiple days of foraging. Each 24 h, we recorded the number of bees that survived from each cage. We also calculated the weight of sucrose and FLU consumed per cage at 24 h intervals, and subsequently calculated the average amount of sucrose solution and FLU consumed per living bee. The volume of sucrose solution consumed (which is directly correlated to pesticide

consumption) was calculated using the density of pure sucrose solution (1.23 mg/µL) and g of sucrose solution consumed. Identical control cages with sucrose solution, but without bees, were maintained at the same incubator conditions to measure the average loss of solution due to evaporation, which was less than 1%. This evaporation was factored into our consumption measurements.

After 3 d of chronic exposure, bee flight performance was tested on flight mills. The flight mills used were the same as described in Tosi *et al.* (2017), which are based upon the design of Smith and Jones (2012). Each flight mill consisted of a magnetically levitated, balanced arm upon which the bee flew within a white paper cylinder with alternating black and white stripes to provide a consistent optic flow environment. We harnessed each bee by gluing a thin 1cm long PFTE tube to its thorax (details in Tosi *et al*. 2017). After harnessing, we rested bees by incubating them individually for 30 min (30°C and 50-80% RH), before testing them on flight mills. We recorded whether bees completed a successful flight or were not able to fly at all. Bees were allowed to fly uninterrupted to exhaustion on the flight mills. For each bee, the longest continuous flight was used to calculate flight duration, distance, and velocity. Thoracic surface temperatures of the bees were measured at four time points: before and after the 30-min rest period and before and after the flight trial. Temperature measurements of the bee thorax were made using an imaging infrared thermography camera (Raytek High-Performance Thermal Imager, ThermoView Ti30). IR thermography is a standard method for measuring bee thoracic surface temperature since this is a non-invasive way for estimating bee thoracic muscle temperature (Nieh & Sánchez 2005; Nieh *et al.* 2006). Measured IR temperatures were calibrated as described in Nieh *et al*. (2006).

Post-flight survival assessment

After flights, bees were placed back in individual cages and fed their respective treatment solutions. We did not remove the harness tubes. We recorded the number of days that each bee survived after their flight trial.

Statistics

We analyzed the flights of 300 bees and survival rates of 1276 bees. We used JMP v10.0 to conduct our statistical analyses and set an alpha value of 0.05 for all tests. Survival analyses were performed using a Proportional Hazards fit model, and are reported using the Likelihood Ratio Chi-square statistic. Nominal logistic regressions were used to test the effect of FLU and sucrose on whether foragers would fly in the flight mill (a nominal variable), categorized as either "successful flight" or "no flight."

We used Analysis of Variance (ANOVA, REML algorithm) to test for effects of FLU and sucrose (both treated as nominal variables) on consumption, flight performance, and ΔT (surface thoracic temperatures corrected for ambient air temperatures). We used residuals analysis to ensure that the data met parametric assumptions. Under flight performance, distance and duration were not normally distributed so were log transformed. For a subset of our flight performance data, we measured bee surface thoracic temperatures. To analyze the effects of T (a continuous, fixed variable) and nominal effects on flight performance, we used Mixed Model Analysis of Covariance (ANCOVA).

Season was included as a fixed factor, but models were split by season if there was a three-way interaction involving season. We used stepwise model simplification, first including all interactions in our models and then removing ones that were not significant (Crawley 2007). Colony was a random effect in ANOVA analyses, but was included as a fixed factor in nominal logistic regressions. To examine our data in greater detail and analyze the interactions of tested variables, we used Tukey Honestly Significant Difference (HSD) tests, which are corrected for

potential Type I error, or ran limited contrast tests based upon visual inspection of our data. Each bee was flown only once. Multiple temperature measurements were made of each bee, but we separately analyzed the effects of FLU and nutrition upon each of these temperatures and ran separate models, one per temperature measurement. Thus, we did not use repeated-measures analyses. We report mean±1 standard error.

RESULTS

Details on most statistical tests are reported in the tables.

High-quality sucrose increased forager survival

We collected 1276 bees from six different colonies and analyzed their mortality during a 3-day chronic exposure period before flight testing (Fig. 1). Both summer and winter foragers had higher survival rates during the chronic exposure period when offered a higher quality sucrose diet (L-R χ^2_1=103.35, p<0.0001). However, there was no significant effect of FLU on survival (p=0.130, Table 1), confirming that FLU doses used in our experiments were sublethal. For control bees, survival rate to day three of the chronic exposure was 72.48±2.78%. Bees exposed to 50% sucrose with 4 ppm FLU had a survival of 69.81±4.46%. The survival rates of bees exposed to low-quality pure sucrose and low-quality sucrose with FLU were 42.95±3.96% and 34.69±4.81% respectively. On average, control bees (50% sucrose, 0 ppm FLU) survived for 2.75±0.03 d, bees that consumed 50% sucrose and 4 ppm FLU survived 2.71±0.06 d, bees that consumed 33% sucrose and 0 ppm survived 2.55±0.06 d, and bees that consumed 33% and 4 ppm FLU survived 2.46±0.07 d.

FLU reduced sucrose consumption and low-quality sucrose increased FLU consumption

Analysis revealed a three-way interaction of season, FLU and nutrition ($F_{1,318.7}$=4.61, p=0.033), so analyses were divided by season to clarify seasonal effects. In both summer and winter, there were significant effects of nutrition ($p\leq$0.001, Table 2). In summer foragers, sucrose solution consumption was higher when the available diet was 33% sucrose versus 50% sucrose (p=0.001). In winter foragers, sucrose solution consumption was also higher in bees that consumed 33% sucrose (p<0.0001).

There was a significant effect of FLU in summer bees ($p<0.0001$), but not in winter bees ($p=0.805$). In summer bees, there was a significant interaction of FLU*nutrition ($p=0.042$). Pairwise comparisons of the summer data (Fig. 2) revealed that FLU significantly decreased sucrose consumption only in the 50% sucrose treatment (Tukey HSD test, $p<0.05$).

In both summer and winter foragers, FLU consumption was significantly higher when the available diet was 33% sucrose as compared to 50% sucrose (summer: $F_{1,150.4}=83.71$, $p<0.0001$; winter: $F_{1,\ 129.5}=14.80$, $p=0.0002$).

FLU decreased the proportion of bees that flew in the winter season

We analyzed the flight performance of 300 bees from six colonies. There was a significant three-way interaction between season, nutrition, and FLU (L-R $\chi^2_1=5.92$, $p=0.015$, Table 3); we therefore divided the data by season for subsequent analyses. There was no effect of FLU upon summer flight success (Table 3). In winter bees, however, FLU significantly decreased the ratio of successful flights to failed flights ($p=0.012$). Specifically, bees that fed on low-quality 33% sucrose and FLU had decreased flight success as compared to the control group (contrast test: L-R $\chi^2_1=10.29$, $p=0.0013$). There was no effect of FLU upon bees that were given the higher-quality 50% sucrose diet (L-R $\chi^2_1=0.03$, $p=0.874$). In the winter, bees that consumed 33% sucrose and 0 ppm FLU failed to fly 2.2% of the time, while the failure rate of bees that consumed 33% sucrose and 4 ppm FLU was 21.3%. Thus, FLU increased the flight-failure rate of winter bees by 9.6-fold as compared to controls.

Seasonality and FLU altered flight performance

Forager bees were flown on flight mills to assess flight duration, velocity, and distance. The interaction of season*FLU exposure significantly influenced flight duration and distance ($p\leq0.046$, Table 4). Bees that were not exposed to FLU flew longer (contrast test: $F_{1,195}=5.17$,

p=0.024) and greater distances (contrast test: $F_{1,191}$=4.07, p=0.045) in the summer than in the winter at both nutrition levels (Fig. 4). When exposed to FLU, there were no significant differences in flight performance between winter and summer bees. Thus, FLU affected flight performance: it eliminated the significant difference between summer and winter bees for flight duration and flight distance (Fig. 4).

FLU interacted with sucrose concentration or season to alter forager thoracic temperatures

Forager thoracic temperatures reflect wing muscle temperatures and therefore play a role in flight performance. Here, we examined the effects of our treatments on ΔT (thoracic temperature corrected for ambient temperature). There was a significant effect of season in all models (p<0.0001, Table 5, Fig. 5). The measurements for $\Delta T_{\text{after harnessing}}$, $\Delta T_{\text{after recovery}}$, and $\Delta T_{\text{after flight}}$ were negative (thorax T below ambient T), while $\Delta T_{\text{before flight}}$ was positive (thorax T above ambient T).

Nutrition provides the calories necessary to power flight muscles, and our results showed significant effects of nutrition on $\Delta T_{\text{before flight}}$ (p=0.008) and on $\Delta T_{\text{after recovery}}$ (p=0.004). For $\Delta T_{\text{before flight}}$, control bees that fed on higher quality food had somewhat higher temperatures than those that fed on lower quality food (overall effect of nutrition, p=0.008). There was also a smaller difference between ambient temperature and thorax temperature for control bees that fed on higher quality food ($\Delta T_{\text{after recovery}}$).

FLU significantly decreased the magnitude of $\Delta T_{\text{after harnessing}}$ in winter bees that fed on 33% sucrose solution. FLU significantly decreased the magnitude of $\Delta T_{\text{after recovery}}$ in summer bees that fed on 33% sucrose solution. $\Delta T_{\text{before flight}}$ was significantly higher in winter than in summer (p<0.0001), but there were no effects of FLU. There were also no effects of FLU upon $\Delta T_{\text{after flight}}$.

Nutrition, FLU, and season affected ΔT in multiple complex ways. However, the main effect of FLU was to slightly increase thorax temperature relative to ambient temperature in bees that fed on 33% sucrose solution (Fig. 5A). The main effect of nutrition was to increase thorax temperatures relative to ambient temperature in bees that fed on 50% sucrose solution (Fig. 5).

Thoracic temperature before flight correlates with flight velocity

Because flight power is directly related to flight muscle temperature, we used absolute thorax temperature, not ΔT, in these analyses. There was a significant interaction of season*FLU* nutrition ($F_{1,199}$=5.56, p=0.019) and we therefore divided our subsequent analyses by season. Average flight velocity correlated positively with $T_{\text{before flight}}$ in the winter (R^2=0.14, p=0.024, Table 6). Maximum flight velocity was weakly though significantly correlated with $T_{\text{before flight}}$ in the summer and winter (R^2=0.08-0.16, $p \leq 0.027$). The interaction of FLU and nutrition, and the interaction between FLU and $T_{\text{before flight}}$ also influenced max velocity ($p \leq 0.025$). Overall, there was a complex interaction of season, FLU, nutrition, and thorax temperature in which thorax temperature was weakly correlated with some measures of flight performance.

DISCUSSION

We found that FLU and nutrition could influence survival, sucrose consumption, flight success, thorax temperature, and flight performance, depending on season. In multiple cases, the effects of FLU were revealed only as an interaction with other factors. Our results demonstrate that FLU, at field-realistic levels, can decrease the proportion of foragers that are able to fly in the winter, and reduce the total flight duration and distance flown by foragers in the summer. We used a standard assay of flight, testing bees on flight mills, to provide a controlled measure of flight. Given our results, studies conducted on the flight of bees in open-field conditions would be desirable to more realistically test the effects of FLU, nutrition, and season.

Survival

Low-quality sucrose diet decreased survival rates in foragers by 30% by the third day of chronic exposure, in both summer and winter. This suggests that future studies should examine the interaction of poor nutrition with other stressors and how this may affect other key behaviors.

Consumption

Foragers given access to 33% sucrose consumed more of the solution than foragers that had 50% sucrose. Because 33% sucrose is less nutritious than 50% sucrose per unit volume, it is reasonable that bees would require more of the 33% solution to meet their daily caloric needs. However, it was interesting that the presence of FLU caused summer bees to consume less sucrose solution than their counterparts exposed to the same concentration of sucrose but without FLU (Fig. 2). Bees may possibly have a natural aversion to FLU, but further studies are required to determine this. In addition to consuming more sucrose solution, bees that were fed 33% sucrose also consumed more FLU than bees that were fed 50% sucrose because both solutions had the same concentration of FLU. This may pose an issue for bees that suffer from malnutrition

because the addition of FLU would expose them to a more extreme level of this stressor compared to bees that are receiving adequate nutrition.

Flight success

A key result is that FLU led to a larger percentage of failed flights in bees that consumed low-quality sucrose (Fig. 3). The 50% high-quality sucrose seemed to be an effective buffer for flight ability even with the presence of FLU. It appears that 33% sucrose was not enough to negate the effects of FLU. Flight success was 19% lower in bees exposed to FLU in low-quality sucrose than bees exposed to pure low-quality sucrose. This is of concern because honey bee colonies require foragers to fly to collect food for the hive (Riley *et al.* 2005), and impairment of flight ability could therefore impair overall colony fitness.

Flight performance

Flight performance (duration, distance, and velocity) was influenced by the interaction of season and FLU (Fig. 4). Flight duration and distance were longer in summer control bees than in winter control bees. Summer bees performed significantly better than winter bees (Fig. 4). However, FLU eliminated seasonal differences, suggesting that FLU reduced the ability of summer bees to fly for longer durations and distances.

Unlike prior studies, we found no clear link between nutrition and flight performance ($p \geq 0.057$). Gmeinbauer *et al*. (1993) showed that honey bee flight speed in a flight mill is positively correlated with the sucrose concentration (within a 1-4 M range) fed to the bees, and Balderrama *et al*. (1992) showed that the metabolic rate of flying bees increased with sugar flow rate. However, we exposed our bees to these sugar concentrations chronically, whereas these other studies provided a single feeding of the tested sugar concentrations. It is possible that chronic feeding over multiple days allowed bees to build up their flight reserves and that the

energetic differences provided by 33% and 50% sucrose solution were not large enough to show difference in flight.

ΔTemperature

We found complex effects of FLU on thoracic temperature. In bees not exposed to FLU, the difference in $\Delta T_{\text{after harnessing}}$ was greater in bees that were fed 33% sucrose rather than 50% sucrose, and the difference in $\Delta T_{\text{after recovery}}$ was greater in the winter than the summer. This suggests that bees allowed to consume high-quality sucrose were less affected by stressful situations such as being harnessed and could maintain a thoracic temperature closer to the ambient air temperature compared to bees that consumed low-quality sucrose. This also suggests that FLU exposure may be an additional stressor that increases the sensitivity of bees in the summer or bees with nutritious food sources, given that the presence of FLU diminishes the significant effects of season and sucrose.

$\Delta T_{\text{after harnessing}}$, $\Delta T_{\text{after recovery}}$, and $\Delta T_{\text{after flight}}$ were negative values while $\Delta T_{\text{before flight}}$ were positive values, showing that bees could raise their thoracic temperatures above ambient air temperature in preparation for flight. Nutrition contributes the necessary energy for activation of flight muscles, and our results suggest that higher quality sucrose leads to higher $\Delta T_{\text{before flight.}}$

Absolute thorax temperature

Increased sucrose content caused increased $T_{\text{before flight}}$, as expected (Nieh & Sánchez 2005), and higher thoracic temperatures correlated with greater flight velocities. Our results align with prior research that showed how food quality—both nectar sugar content (Nieh & Sánchez 2005) and pollen protein concentration (Nieh *et al.* 2006)—increased the thoracic temperature of foragers. It has been hypothesized, but not previously shown, that these higher thoracic temperatures resulting from higher quality food should increase the ability of bees to fly and

thereby retrieve this food. In our experiments (Table 6), we showed that increase in thoracic temperature could result in faster flights. However, we note that these results are somewhat weak (low correlation coefficients), and further studies are called for.

Seasonal effects

Winter and summer bees differ in multiple ways. It is expected that more summer bees are employed in collecting food than winter bees. However, it also true that winter bees should have greater longevity and be more resistant to multiple stressors (Winston 1987, Ribbands 1953), including pesticides (Decourtye *et al.* 2003). How do our results compare with these expectations? As anticipated, summer bees performed better than winter bees in terms of flight duration and distance (Fig. 4). We predicted winter bees to be more resistant to FLU, but in our study FLU reduced the flight success of winter bees and not summer bees (Fig. 3). It is possible that we got our results because winter bees are subject to greater stresses from the climate (Winston 1987). In fact, most colony losses occur in the winter (van Engelsdorp *et al.* 2008). Thus, future studies should examine the effects of FLU on overwintering bees in moderate climates where some level of flight occurs throughout the year. This is true of honey bee colonies maintained, for example, in the southern USA and in multiple tropical and subtropical areas around the world.

Summary

Our results suggest that the effects of FLU interact with season and nutrition in a complex way. Moreover, the effects of FLU can be somewhat subtle. These results align with recent research showing that this new butenolide insecticide has little or no adverse effects on honey bees at recommended levels of use (Nauen *et al.* 2015; Jeschke *et al.* 2015; Campbell *et al.* 2016). However, FLU at sublethal and field-realistic levels impaired the ability of bees to fly

(flight success), reduced consumption of artificial nectar, and impaired flight duration and distance. Additional studies should therefore be conducted in different seasons to assess the safety of FLU with other stressors on pollinators. Future studies may examine how FLU affects the success of foragers returning to the nest and examine synergistic effects of FLU, pathogens, and common xenobiotics that may affect honey bee health.

APPENDIX

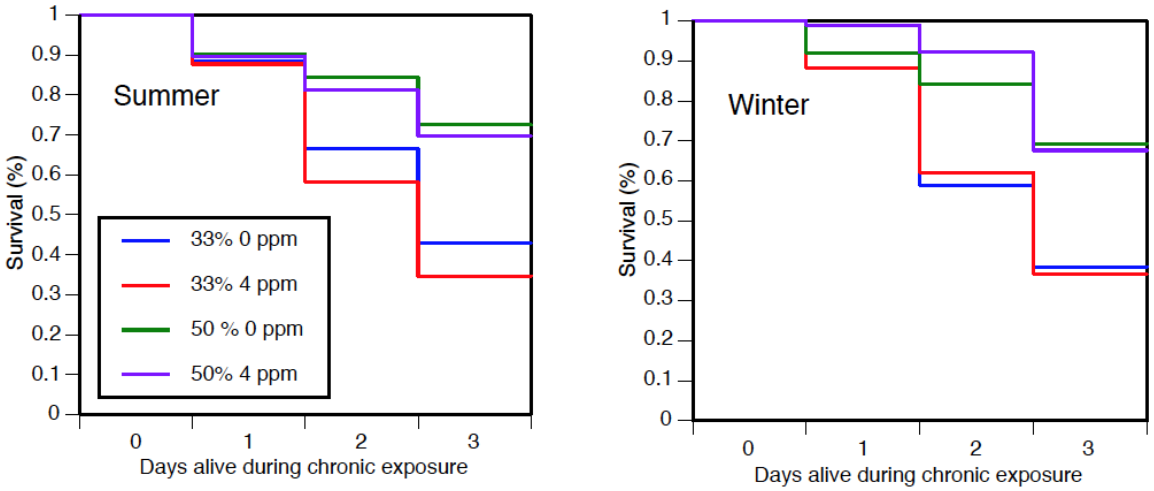

Figure 1. Effects of nutrition and FLU on honey bee survival during chronic FLU exposure. The survival curves are split into summer and winter to display seasonal variability, but the survival analysis (Proportional Hazards Fit) was run with season as a fixed factor. In both summer and winter, foragers that fed on 50% sucrose had significantly higher survival than bees that fed on 33% sucrose ($p<0.0001$). There was no significant effect of pesticide ($p=0.130$). However, there was an effect of season ($p=0.006$) as seen in general differences in the survival curves between summer and winter.

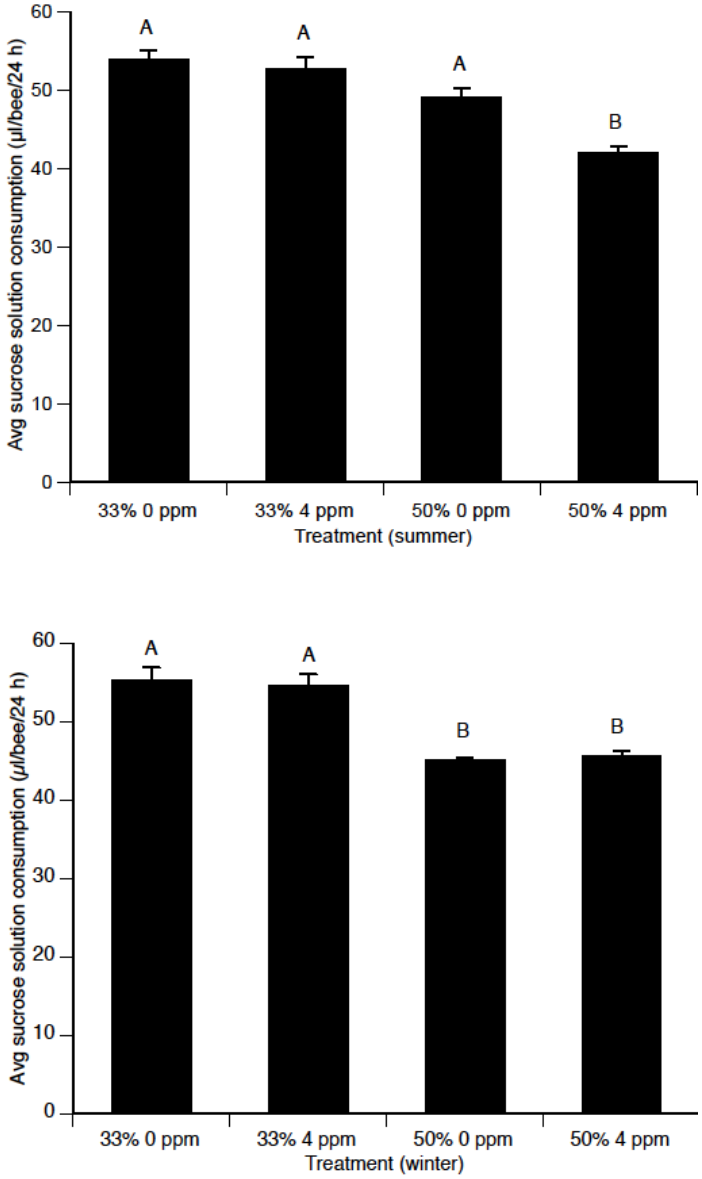

Figure 2. Effects of sucrose concentration and FLU on sucrose solution consumption (µl/bee/day). Analysis revealed a three-way interaction of season, FLU and nutrition (p=0.033), so subsequent analyses were split by season. Lower quality food, 33% sucrose, led to greater consumption of sucrose solution ($p \leq 0.001$). Subsequently, honey bees consuming 33% sucrose (w/w) ingested more FLU than bees consuming 50% sucrose in the summer ($F_{1,150.4}$=83.71, p<0.0001) and the winter ($F_{1,\ 129.5}$=14.80, p=0.0002). There was an effect of FLU in the summer, when the combination of high quality sucrose and FLU exposure caused significantly lower levels of solution consumption compared to all other treatments. In the winter, only sucrose concentration affected consumption. Different letters indicate significant differences (Tukey HSD test, p<0.05).

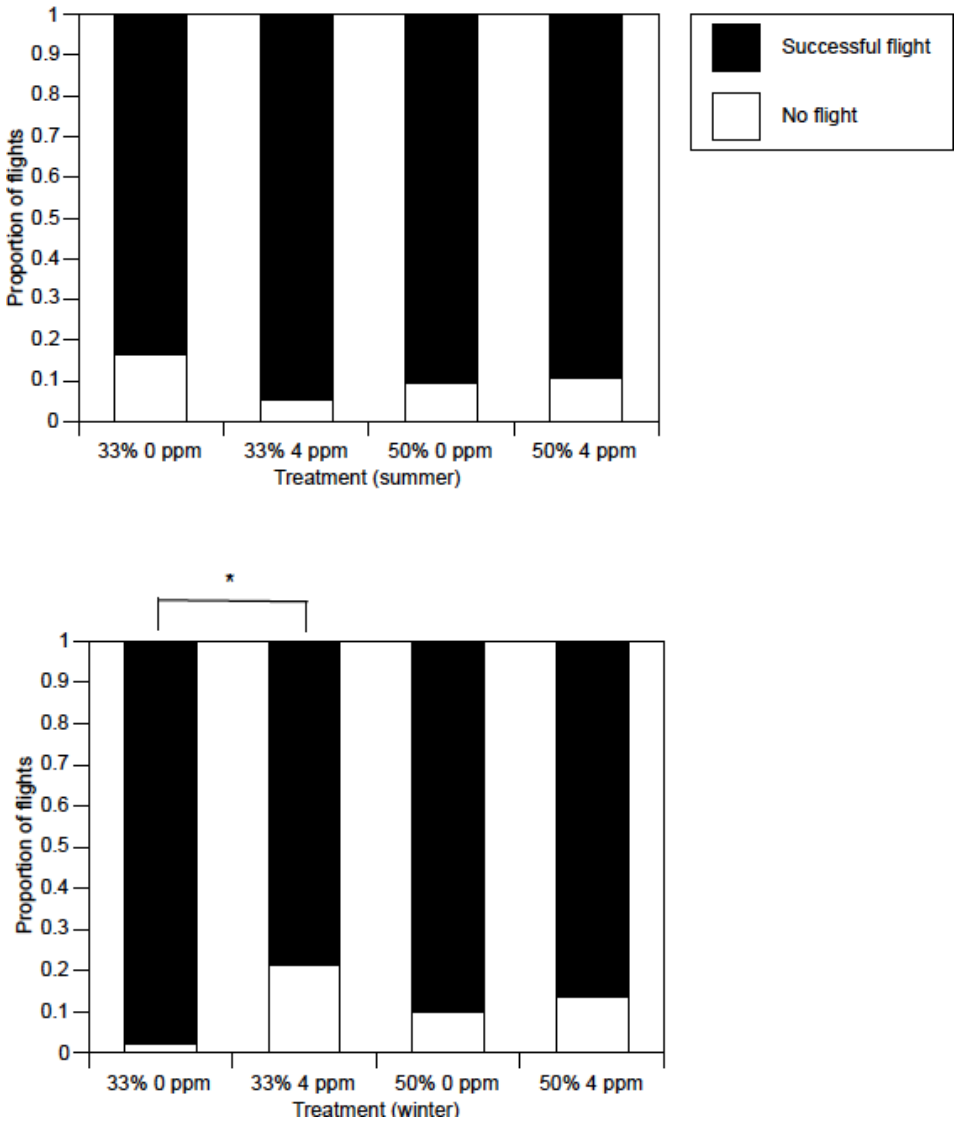

Figure 3. Effects of sucrose concentration and FLU on the ability of bees to fly in the flight mill. There was a significant three-way interaction between season, nutrition, and FLU (p=0.015) and thus we divided the data by season for subsequent analyses. FLU exposure significantly decreased flight success in winter foragers (winter FLU effect: p=0.012), but not in summer foragers (Table 4). Based upon visual inspection of the data, we conducted two contrast tests of the winter data. FLU significantly decreased successful flights in the winter when bees were fed a low-quality 33% sucrose diet (contrast test, p=0.0013, see star and connector bar), but not when fed the higher-quality 50% sucrose diet (contrast test, p=0.874).

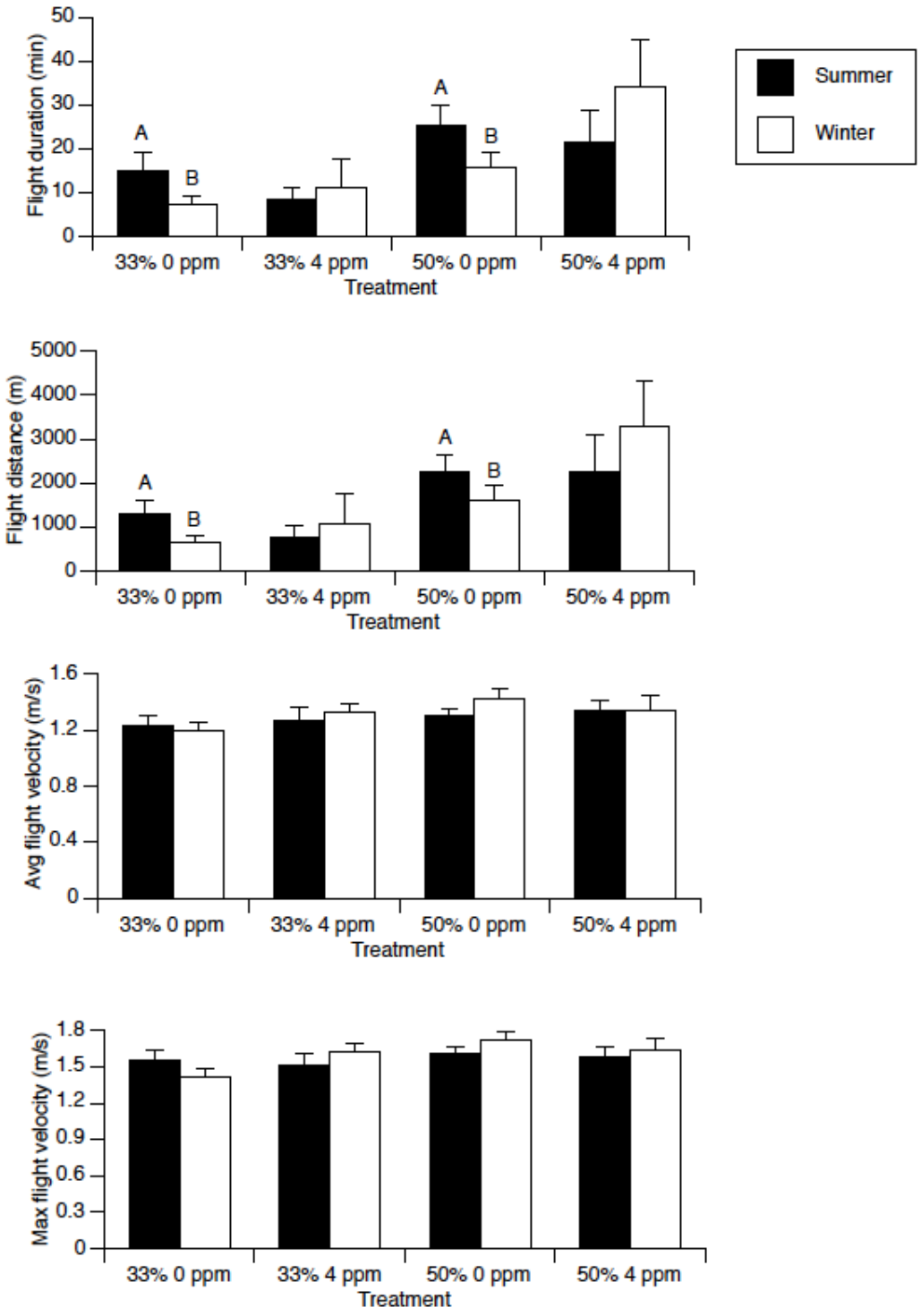

Figure 4. Effects of sucrose concentration and FLU exposure on honey bee flight performance. The interaction of season and FLU significantly affected flight duration and distance. Summer bees with no FLU exposure flew longer durations and greater distances than winter bees with no FLU exposure (contrast tests, $p<0.05$). However, FLU treatment eliminated these seasonal differences. Different letters indicate significant differences.

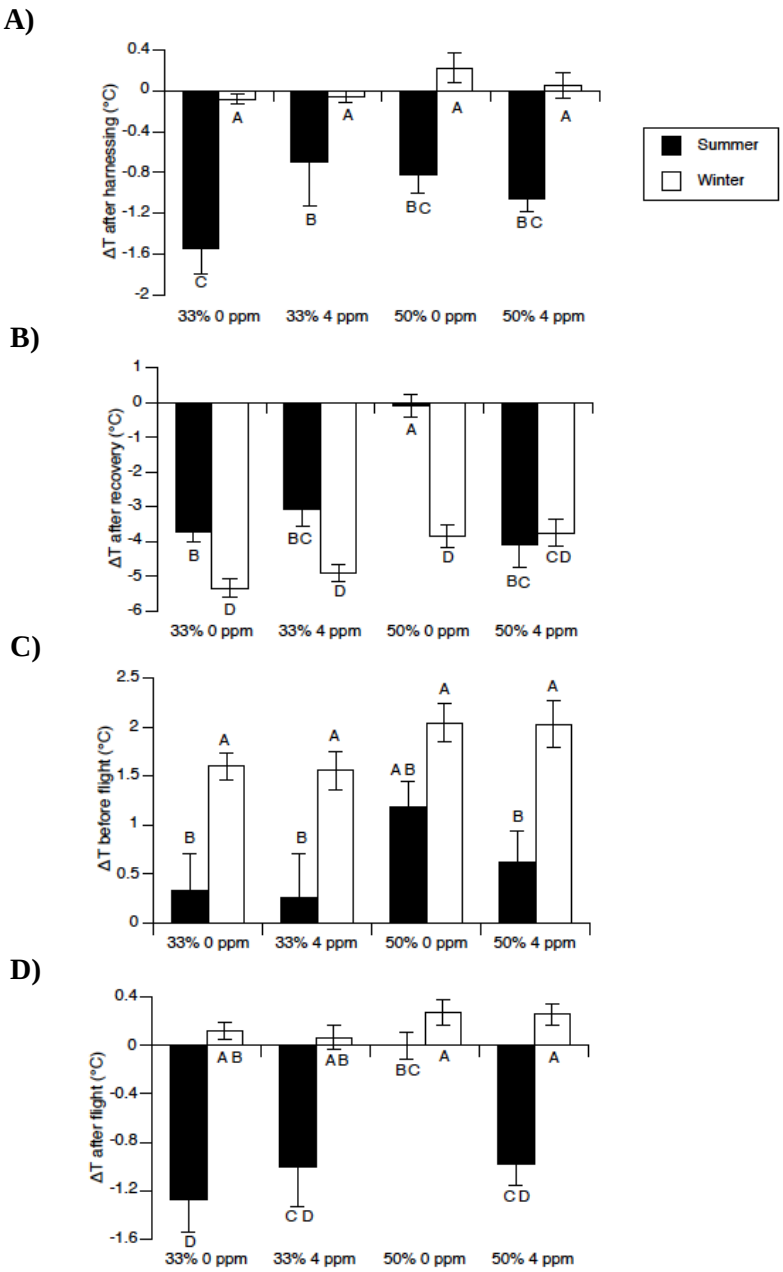

Figure 5. Effects of FLU and nutrition on forager thoracic temperatures ΔT (°C). ΔT is the difference between the surface thoracic temperature and the ambient air or incubator temperature, as appropriate. Analyses were run with season as a fixed factor, but graphs are separated by season to show seasonal differences. **A)** $\Delta T_{\text{after harnessing}}$ was greater in summer than in winter ($p<0.0001$). FLU significantly decreased the magnitude of $\Delta T_{\text{after harnessing}}$ in summer bees that fed on 33% sucrose solution. **B)** FLU significantly increased the magnitude of $\Delta T_{\text{after recovery}}$ in summer bees that fed on 50% sucrose solution. **C)** $\Delta T_{\text{before flight}}$ was significantly higher in winter than in summer ($p<0.0001$). but there were no effects of FLU. Bees that fed on 50% sucrose that higher $\Delta T_{\text{before flight}}$ than bees that fed on 33% sucrose. **D)** $\Delta T_{\text{after flight}}$ was significantly different between seasons ($p<0.0001$). There were no effects of FLU. Different letters indicate significant differences (Tukey HSD test: $p<0.05$).

Table 1. Effects of FLU and nutrition on forager survival before flight trial (3-day chronic exposure period). We report a Proportional Hazards fit survival analysis. Non-significant interactions were removed from the model. $N_{\text{bees before flight trials}} = 1276$.

Parameter	**Tested variable**	**DF**	**L-R ChiSquare**	***P*-value**
survival before flight	FLU	1	2.29	0.130
	Nutrition	1	103.35	<0.0001
	Season	1	7.64	0.006
	Colony	10	121.95	<0.0001

Table 2. Effects of nutrition (sucrose concentration) and FLU on the volume of sucrose solution consumption. Because of a three-way interaction between season, FLU, and sucrose concentration ($F_{1,318.7}$=4.61, p=0.033), we divided our results by season to facilitate analysis. Non-significant interactions were removed from the model.

Parameter	Season	Model fit (R^2)	Colony effect (%)	Tested variable	DF numerator	DF denominator	*F* ratio	*P*-value
Sucrose consumption	Summer	0.420	41.66	FLU	1	177.7	19.03	<0.0001
				Nutrition	1	182.9	10.85	0.001
				FLU * Nutrition	1	178.8	4.19	0.042
	Winter	0.315	8.96	FLU	1	140.5	0.06	0.805
				Nutrition	1	134.2	39.60	<0.0001

Table 3. Effects of FLU and nutrition on flight success. Because of a three-way interaction between season, FLU, and sucrose concentration (p=0.015), we divided our results by season to facilitate analysis. Winter bees had a larger percentage of flight failure when subject to FLU exposure. Non-significant interactions were removed from the model ($N_{success, summer}$=173; $N_{failure, summer}$=21; $N_{success, winter}$=127; $N_{failure, winter}$=17).

Parameter	**Season**	**Tested variable**	**DF**	**L-R ChiSquare**	***P*-value**
Flight success	Summer	FLU	1	0.01	0.925
		Nutrition	1	0.14	0.705
		Colony	8	10.93	0.206
	Winter	FLU	1	6.31	0.012
		Nutrition	1	0.19	0.661
		Colony	6	6.38	0.382

Table 4. Effects of FLU and nutrition on bee flight. FLU, sucrose concentration, and season were included as fixed factors. Non-significant interactions were removed from the model.

Parameter	Model fit (R^2)	Colony effect (%)	Tested variable	DF numerator	DF denominator	*F* ratio	*P*-value
Flight duration	0.079	3.23	FLU	1	288.1	0.16	0.687
			Nutrition	1	215.2	3.28	0.071
			Season	1	212.7	0.56	0.455
			Season*FLU	1	289.1	4.82	0.029
Flight distance	0.076	3.42	FLU	1	287.8	0.21	0.647
			Nutrition	1	212.3	3.66	0.057
			Season	1	210.6	0.39	0.533
			Season*FLU	1	289.0	4.01	0.046
Flight avg. velocity	-0.012	0.00	FLU	1	285.9	0.89	0.346
			Nutrition	1	10.22	2.26	0.163
			Season	1	13.79	1.19	0.294
Flight max. velocity	0.042	2.68	FLU	1	269.2	0.38	0.538
			Nutrition	1	138.4	3.44	0.066
			Season	1	107.5	0.01	0.937

Table 5. Effects of FLU and nutrition on forager thoracic temperature ΔT (°C). All insignificant interactions were removed from the models. We calculated ΔT, the difference between the surface thoracic temperature and the ambient air temperature. Non-significant interactions were removed from the model.

Parameter	Model fit (R^2)	Colony effect (%)	Variable tested	DF Numerator	DF Denominator	*F* ratio	*P*-value
after harnessing ΔT (°C)	0.366	11.81	FLU	1	228.9	0.28	0.596
			Nutrition	1	226.3	3.42	0.066
			FLU*Nutrition	1	228.4	4.77	0.030
			Season	1	215.0	87.16	<0.0001
after 30 min recovery ΔT (°C)	0.702	63.20	FLU	1	223.4	1.02	0.313
			Nutrition	1	225.8	8.35	0.004
			Season	1	226.4	83.14	<0.0001
			FLU*Season	1	223.7	8.03	0.005
before flight ΔT (°C)	0.218	7.47	FLU	1	229.1	0.40	0.526
			Nutrition	1	222.0	7.28	0.008
			Season	1	198.3	46.62	<0.0001
after flight ΔT (°C)	0.391	24.60	FLU	1	227.4	0.10	0.746
			Nutrition	1	230.0	3.49	0.063
			Season	1	228.3	98.04	0.0001

Table 6. Effect of thoracic temperature, FLU and sucrose concentration on flight performance. There was a significant interaction of season*FLU*nutrition and we therefore analyzed the data split by season. Because flight power is directly related to flight muscle temperature, we used absolute thorax temperature, not ΔT in these analyses. Only flight average and maximum velocity shown because there were no significant results for flight duration and distance. Non-significant interactions were removed from the model.

Parameter	Season	Model fit (R^2)	Colony effect (%)	Tested variable	DF numerator	DF denominator	*F* ratio	*P*-value
Flight avg. velocity	Summer	0.03	0	FLU	1	81	0.11	0.737
				Nutrition	1	81	0.00	0.994
				Before flight T	1	81	2.55	0.114
	Winter	0.10	0	FLU	1	120	1.79	0.183
				Nutrition	1	120	3.66	0.058
				Before flight T	1	120	5.24	0.024
Flight max. velocity	Summer	0.06	0	FLU	1	81	0.18	0.669
				Nutrition	1	81	0.00	0.991
				Before flight T	1	81	5.08	0.027
	Winter	0.16	0	FLU	1	118	0.37	0.545
				Nutrition	1	118	2.65	0.106
				Before flight T	1	118	8.35	0.005
				FLU*Nutrition	1	118	5.14	0.025
				FLU*Before flight T	1	118	5.81	0.018

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