ABSTRACT

KO, ALEXANDER EUGENE. The impacts of Diet and Insecticide Resistance on German cockroach Foraging Behavior, Mating Success, and Bait Performance (*Blattella germanica*). (Under the direction of Dr. Jules Silverman and Dr. Coby Schal).

The German cockroach (*Blattella germanica*) is a cosmopolitan urban pest, capable of producing allergens and vectoring pathogenic microorganisms. The most common method of controlling German cockroach populations is through the use of bait formulations, which largely consist of a nutrient base and toxicant. A characteristic of many modern bait formulations is secondary kill, whereby insect residues from bait-affected cockroaches are fed upon secondarily by conspecifics, often relatively sedentary early instar nymphs which use the fecal material as a source of nutrition. Two factors that influence the biology and control of this insect are insecticide resistance and the macronutrient composition of the diets available.

In the first study, we investigated the effects of insecticide resistance in a field collected strain of *Blattella germanica* to three active ingredients (fipronil, hydramethylnon, indoxacarb) on primary kill and secondary kill. Our results revealed several novel findings: 1) First evidence of hydramethylnon resistance in any insect; 2) extremely high levels of indoxacarb resistance; and 3) reduced secondary mortality in a multi-insecticide resistant field-collected strain. We suggest that while secondary mortality is considered to be advantageous in cockroach interventions, the ingestion of sublethal doses of AI in cockroach nymph populations may select for high degrees of insecticide resistance by increasing the frequency of AI resistance alleles within the population.

In the second study, we investigated the effect of diet quality on bait performance. We created artificial diets that varied in macronutrient content and sugars that were either absent toxicant ("diet") or contained toxicant ("bait). We provided these diets and baits to both glucose-accepting and glucose-averse cockroaches, and we hypothesized that the interaction between diet composition and bait composition would affect the survival of adult males as well as first instar nymphs exposed to excretions produced by these males. Both primary and secondary kill were affected by the diet and bait combinations to which the adults were exposed. We suggest that disparities between the nutritional quality of baits and the foods that are naturally available could have profound consequences in the management of German cockroach infestations.

In a third study, we examine the fitness-related costs of insecticide resistance with a field-collected strain of *B. germanica*. Under optimal and sub-optimal nutritional conditions, we compared developmental parameters of three strains of cockroach differing in insecticide resistance to indoxacarb. We find that both indoxacarb resistance and poor nutritional condition increased development time to the adult stage, lowered body size and mass, and decreased survival probability of nymphs developing to the adult stage. This study exemplifies the fitness costs of indoxacarb resistance, readily apparent under low nutrient conditions.

In a fourth study, we examined the effects of short and long foraging arenas on specific nutrient consumption, growth and development, and reproductive speed. We found that nymphs in long foraging arenas consumed more protein relative to carbohydrate and gained greater structural mass as a result. Differential

consumption of adult females in long foraging arenas also resulted in greater mass gain and faster reproductive speed than those in short foraging arenas.

In the fifth study, we examine the effect of diets that vary in macronutrient composition on male mating success. During copulation, males provision their spermatophore with stored urates, which can be utilized by the female for the development of offspring. Here we test the hypothesis that protein-deficient females assess male fitness through their ability to contribute urates, and find that while high carbohydrate diets increase a male's mating success, males given the opportunity to feed from a choice of diets experience slightly higher mating success, suggesting that males select a dietary macronutrient blend that improves their mating success.

The Impacts of Diet and Insecticide Resistance on German cockroach Foraging Behavior, Mating Success, and Bait Performance (*Blattella germanica*)

by
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DEDICATION

To my family and friends for their support.

BIOGRAPHY

Alexander Eugene Ko was born and raised in Illinois. He obtained his Bachelors of Science Degree from Illinois State University in Biology Education, and his Masters of Science Degree from Winthrop University in Biology.

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Insecticide resistance and diminished secondary kill performance of bait formulations against German cockroaches (Dictyoptera: Blattellidae)

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Abstract

BACKGROUND:

Bait formulations are considered the most effective method for reducing German cockroach infestations. An important property of some bait formulations is secondary kill, whereby active ingredient is translocated in insect-produced residues throughout the cockroach population, especially affecting relatively sedentary early instar nymphs.

RESULTS:

Blattella germanica was collected from a location where baits containing hydramethylnon, fipronil, or indoxacarb became ineffective, and these AIs were topically applied to adult males. Results revealed the first evidence for hydramethylnon resistance, moderate resistance to fipronil and extremely high resistance to indoxacarb. Insecticide residues excreted by field-collected males that ingested commercial baits effectively killed nymphs of an insecticide-susceptible laboratory strain of *B. germanica* but failed to kill most nymphs of the field-collected strain.

CONCLUSIONS:

We report three novel findings: 1) The first evidence for hydramethylnon resistance in any insect; 2) extremely high levels of indoxacarb resistance in a field population; and 3) reduced secondary mortality in an insecticide-resistant field-collected strain of *B. germanica*. We suggest that while secondary mortality is considered to be advantageous in cockroach interventions, the ingestion of sublethal doses of AI by

nymphs may select for high insecticide resistance by increasing the frequency of AI resistance alleles within the population.

Keywords: *Blattella germanica*, fipronil, indoxacarb, hydramethylnon, insecticide resistance, bait, secondary kill

1 Introduction

The German cockroach (*Blattella germanica*) is a widespread urban pest of significant health concern, mainly because it produces asthma-triggering allergens[1,2] and can vector pathogenic microorganisms.[3-5] It is generally recognized that the most effective way to control German cockroach populations is with bait formulations.[6] These products include nutrients that stimulate feeding and a toxic active ingredient (AI). The targeted nature of bait formulations reduces insecticide exposure to humans and their pets.[6,7] Several bait formulations have secondary[8,9] and even tertiary[10] kill properties through various mechanisms, including coprophagy,[8] cannibalism,[11] and emetophagy.[12] Deposition of insecticide-containing feces within harborages and consumption of feces by early instar nymphs via coprophagy can make the insecticide more accessible to the cockroach population.[8,13-15]

Though bait formulations are largely effective, several *B. germanica* field populations have been reported to be resistant to some bait AIs (sulfluramid;[16] fipronil;[17-19] indoxacarb;[20] abamectin;[18,19] imidacloprid[19]). Hydramethylnon[21] has been a very effective AI in bait products against the German cockroach. Despite its longstanding use since the 1980s, no hydramethylnon-resistant populations of any

insect have been found, and the highest resistance ratio (RR) reported in the German cockroach was 1.5.[16,22-25]

In August 2012, we collected *B. germanica* from an apartment in Puerto Rico where performance of Advion® (AI – indoxacarb), Maxforce FC Magnum® (AI – fipronil) and Maxforce Pro Roach Killer® (AI – hydramethylnon) gel baits was poor. We conducted both laboratory bait efficacy studies and topical assays with fipronil, indoxacarb, and hydramethylnon to determine whether insecticide resistance in this strain (PR 712) might explain, in part, the observed poor bait performance. Moreover, we investigated secondary kill in this strain. All reports to date on cockroach secondary kill had been performed with longstanding insecticide-susceptible laboratory colonies. Yet, the efficacy of baits may be further compromised in resistant populations by poor secondary kill performance. Our goals were to 1) characterize the collected field strain for resistance through topical application of AI and ingestion of formulated bait and 2) compare the toxicity of adult excreta to nymphs from the field-collected strain and from a laboratory-susceptible strain of *B. germanica*.

2 Experimental Methods

2.1 Insect strains and rearing conditions

We compared two *Blattella germanica* strains: (1) Orlando Normal, an insecticide susceptible strain maintained in the laboratory for over 70 years, (2) PR-712, collected in August 2012 from a single apartment in Monseratte Tower 1,

Carolina, Puerto Rico. The cockroach population in this unit could not be controlled with a range of commercial bait products. We propagated newly collected PR 712 for 2-3 generations then allocated these insects to four treatments: (1) unselected, (2) fipronil-selected (Maxforce FC Magnum gel bait), (3) indoxacarb-selected (Advion gel bait), and (4) hydramethylnon-selected (Maxforce Pro Roach Killer). Insecticide selection was accomplished by placing approximately two grams of bait in a rearing container for three days then removing any remaining bait. After bait exposure, living insects were moved to a clean container. Food (Purina 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO, USA) and water were provided *ad libitum*. This process was repeated every 2 months for 2 years, after which time the experiments detailed below were performed. Laboratory conditions for insect rearing and all experiments were 25 ± 1°C), 37 ± 5% RH and 12:12 h L:D.

2.2 Topical application of insecticides

Technical insecticides were serially diluted with acetone and 0.5 μL of a dilution was applied to the ventral surface of the cockroach between the metacoxae with a repeating micro-pipette (Hamilton Company, Reno, NV, USA). At least 30 individuals were treated with each concentration. Following treatment, cockroaches were maintained in three groups of ten in 10 cm diameter petri dishes (Fisher Scientific, Pittsburgh, PA) and provisioned with rat chow and water. Cockroaches topically treated with fipronil or indoxacarb were monitored for mortality daily for 2 days; those treated with hydramethylnon for 5 days. The greatest range of mortality for the AIs tested at given doses occurred at these two time points. Insects that

could not right themselves within 30 s when flipped, and would exhibit erratic appendage movements were considered dead. Values for LD_{50} and LD_{90}, and their respective fiducial limits, were estimated from Probit analysis in Polo Plus (LeOra Software Company, Petaluma, CA, USA).[26]

2.3 Effect of bait formulations on adult male survival

We provided male German cockroaches ~0.5 g of one of three baits in a vial cap (Maxforce FC Magnum, 0.05% Fipronil; Advion, 0.06% Indoxacarb; Maxforce Pro Roach Killer, 2.15% Hydramethylnon), plus rodent chow and water for one week. The cockroaches were housed in glass jars (88 mm diameter, 95 mm height), with the inside rim coated with a thin layer of petroleum jelly/mineral oil to prevent escape. We recorded mortality daily and removed dead cockroaches. Five replicates, with 20 insects per replicate were performed for each of the treatments.

2.4 Secondary toxicity to nymphs

After 1 week of bait exposure, all adults and bait were removed from the jars. We then placed 20 first-instar nymphs in each jar: ten of an insecticide-susceptible orange-body variant of Orlando Normal,[27] plus ten PR-712 nymphs from the same selection regime cohort initially evaluated in the bait experiment with adult males. The orange-body variant enabled us to distinguish effects on insecticide-susceptible and insecticide-resistant nymphs exposed to the same residues (feces, regurgitate), and thus served as a within-jar control. As a control for this color variant, both black-body (wild type) and orange-body Orlando Normal nymphs were exposed to

deposits produced by black-body adult male Orlando Normal. Rodent chow and water were provided *ad libitum*.

2.5 Statistical analysis

LD_{50} and LD_{90} values for each strain-AI pair were calculated and compared using the lethal dose ratio test, whereby LD_{50} and LD_{90} values are significantly different from one another if the upper and lower 95% confidence intervals of the ratio do not contain 1 (PoloPlus program, LeOra Software Company, Petaluma, CA, USA).[26] We used a log-rank test to compare strains in the bait primary and secondary kill assays. A Sidak-adjustment was used to account for multiple-comparisons in primary kill (SAS 9.3, SAS Institute, Cary, NC). No adjustment was needed for comparisons of secondary kill because only two strains were compared. Resistance ratios (RR) were calculated by dividing the LD_{50} and LD_{90} values of the PR-712 strain by the respective LD_{50} and LD_{90} values of the Orlando Normal susceptible strain.

3 Results

3.1 Topical application of insecticides

Acetone alone did not cause any mortality. The PR-712 strain was significantly more resistant to fipronil, indoxacarb, and hydramethylnon than the Orlando Normal susceptible strain (LD_{50} RR: 5.60, 23.21, 3.89; LD_{90} RR: 9.78, 391.3, 8.74, respectively; Table 1). Continued lab selection of PR-712 increased

fipronil, indoxacarb, and hydramethylnon resistance (LD_{50} RR: 15.92, 13,375, 19.31; LD_{90} RR: 20.20, ~54,619, 350.9, respectively; Table 1). LD_{90} could not be accurately estimated for the PR-712 lab-selected cockroaches treated with indoxacarb, because only 16% of the individuals died at the highest topical dose (150 µg 0.5 μL^{-1}), so an approximation was made based on $\log_{10}$ dose and probit value regression.

3.2 Effect of bait formulations on adult male mortality

Unselected PR-712 males survived longer than Orlando Normal males when exposed to hydramethylnon bait (Log rank test: $\chi^2 = 14.6813$, $P = 0.0004$) but not Maxforce FC Magnum ($\chi^2 = 0.6850$, $P = 0.7924$) or Advion ($\chi^2 = 0.5389$, $P = 0.8450$; Fig. 1).

Continuous laboratory selection with each bait extended the survival of PR-712 relative to Orlando Normal (fipronil bait: $\chi^2 = 8.3912$, $P = 0.0113$, indoxacarb: $\chi^2 = 42.6871$, $P < 0.0001$, hydramethylnon: $\chi^2 = 127.2$, $P < 0.0001$) and unselected PR-712 (indoxacarb bait: $\chi^2 = 29.4652$, $P < 0.0001$, hydramethylnon: $\chi^2 = 34.2846$, $P < 0.0001$; Figs 1B and C) but not for fipronil ($\chi^2 = 4.2812$, $P = 0.1112$; Fig. 1A).

3.3 Secondary toxicity to nymphs

The orange-body within-jar controls did not differ from the black-bodied insecticide-susceptible strain when exposed to secondary excretions from black-body Orlando Normal adult males fed fipronil- or indoxacarb-containing cockroach

bait (χ^2 = 0, P = 1.0; χ^2 = 2.0222, P = 0.1550; Figs 2A and B respectively) but the two lab susceptible genotypes differed in their secondary response to hydramethylnon (χ^2 = 6.4451, P = 0.0111; Fig. 2C). Nevertheless, the absolute differences between black and orange body cockroaches were relatively minor and justified the use of the latter in subsequent assays.

In all subsequent assays nymphs were exposed to residues of adults of the same strain, so different amounts of residues might have been available in different treatments. Therefore, comparisons should be limited mainly to the two strains co-habiting the same jar. Nymphs of the PR-712 unselected strain survived significantly longer than nymphs of the Orlando Normal susceptible strain when exposed to secondary excretions from PR-712 unselected adults that had been fed fipronil-, indoxacarb-, or hydramethylnon-containing bait (log-rank test: χ^2 = 12.2360, P = 0.0005; χ^2 = 27.2406, P < 0.0001; χ^2 = 40.4296, P < 0.0001; Figs 2D, E and F, respectively).

Laboratory selection with baits further decreased mortality of PR-712 nymphs exposed to residues of adult males; these nymphs always survived significantly longer than Orlando Normal nymphs, regardless of bait (Maxforce FC Magnum: χ^2 = 87.7925, P < 0.0001; Advion: χ^2 = 106.5, P < 0.0001; Hydramethylnon bait: χ^2 = 38.8107, P < 0.0001; Figs 2G, H and I, respectively).

4 Discussion

The PR-712 strain was collected from an apartment where repeated treatments with insecticidal baits failed to provide adequate cockroach control. We

report three novel findings from experiments with this field-collected strain of *B. germanica*: 1) The first evidence for hydramethylnon resistance in any insect, 2) rapid elevation in both hydramethylnon and indoxacarb resistance in response to selection, and 3) reduced hydramethylnon, indoxacarb, and fipronil secondary mortality in nymphs, suggesting a novel mechanism of selection for insecticide resistance.

4.1 Resistance to active ingredients

Our findings with topical applications of insecticides indicate that control failures were, at least in part, attributable to resistance to a broad-spectrum of AIs. Cockroaches have developed resistance to every organic insecticide within several years of intensive usage, going back to DDT, regardless of its formulation.[6] Resistance has been documented even to the most recent introductions of new AIs.[25] Surprisingly, however, despite its widespread and intensive usage in commercial cockroach baits for over 30 years, resistance to hydramethylnon has remained low, with a RR less than 1.5.[16,22-25] The field-collected PR-712 strain exhibited RR_{50} and RR_{90} of 4-fold and 9-fold, respectively, and after artificial selection these values increased to 19-fold and 351-fold, respectively (Table 1). This rapid increase in resistance following two years of continuous artificial selection indicates that the allele(s) underlying hydramethylnon resistance were present in this population and selection elevated their frequency. Moreover, stability of hydramethylnon resistance in the PR-712 strain suggests that reversion to susceptibility in the absence of selection would be slow. Although specific records of

hydramethylnon use are unavailable, hydramethylnon-based baits presumably selected on this population sometime prior to 2010.

The PR-712 population was exposed to intensive applications of Advion (indoxacarb) and Maxforce FC Magnum (fipronil) gel baits between 2010 and 2012, and not surprisingly, this strain exhibited resistance to both AIs (Table 1), showing that this is a multiresistant strain. As with hydramethylnon, topical assays revealed that continuous artificial selection on PR-712 significantly increased resistance (RR_{50}) to fipronil from six- to 16-fold and to indoxacarb from 23- to > 10 000-fold compared to the susceptible strain. Such high levels of resistance, based on topical applications, would be expected to impede pest control efforts.

However, bait formulations may be efficacious even in moderately resistant populations. The difference in mortality between insecticide-susceptible Orlando Normal and PR-712 was much less in bait feeding tests than from topical application, as most adult PR-712 died by the end of the bait feeding trials. The disparity between topical application and ingestion results may be related to two major issues: First, many modern bait AIs are much more toxic by ingestion than when topically applied, and some are activated by gut enzymes. Gondhalekar *et al.*[25] reported a lower RR to indoxacarb when a *B. germanica* field strain ingested the AI compared to topical exposure, possibly a consequence of post-ingestive activation.[28] Secondly, ingestion can deliver a massive dose of AI that often can overcome low to moderate resistance. For example, fipronil's LD_{50} was 2.12 ng per Orlando Normal male (LD_{90} = 3.18 ng). Ingesting 2.5 mg of bait (0.05% AI), the typical daily intake of adult males,[29] would deliver 1,250 ng fipronil, or almost 600-

fold the LD_{50} and nearly 400-fold the LD_{90}. This amount of AI would be sufficient to overcome the 16-fold resistance even of our artificially selected PR-712 strain. Likewise, the corresponding Orlando Normal estimates for hydramethylnon are: topical LD_{50} = 1.0 µg (LD_{90} = 2.9 µg); bait (2.15% AI) would deliver 53.8 µg AI, which is much more than necessary to kill the hydramethylnon selected PR-712 cockroaches. For indoxacarb, however, these estimates reveal that ingestion of even large amounts of AI are not likely to overcome indoxacarb resistance. The topical LD_{50} for Orlando Normal is 201 ng indoxacarb per male (LD_{90} = 323 ng); the Advion bait (0.06% AI) would deliver 1500 ng AI, ~7.5-fold the LD_{50} dose. Thus, it is unlikely that the ingested dose would overcome a RR_{50} of 23.2 in the unselected strain, and >10 000 in the artificially selected PR-712 strain. We emphasize two important points regarding comparisons of topical applications and ingestion: First, we provided bait in excess, allowing continued *ad libitum* ingestion for the 7 day duration of the experiment. Thus, whereas faster-acting AIs (e.g., fipronil, indoxacarb) incapacitate the cockroach and probably limit it to a single meal, slower acting AIs (e.g., hydramethylnon) may allow multiple meals – and more AI ingestion – before death. Secondly, these comparisons assume similar toxicodynamics for ingested and topically applied AIs, but more of the ingested than surface-applied AI is expected be metabolized *in vivo*, leaving a smaller percentage of ingested AI to reach the target site, compared to topical application where AI bypasses the harsh digestive tract. Nevertheless, the huge amounts of ingested AIs compared to topically applied AIs, discussed above, are expected to compensate for any losses due to AI metabolism. Overall, these estimates predict that under field conditions,

where cockroach populations are high and bait is often limited, highly or moderately resistant cockroaches may not ingest sufficient bait to succumb to a lethal dose of insecticide.

4.2 Secondary kill of nymphs

Our secondary-kill assay was designed to expose both Orlando Normal and PR-712 first instar nymphs to equal amounts of insecticide residues produced by either Orlando Normal or PR-712 males. To distinguish these two cohabiting strains, we used orange-body mutants of the susceptible Orlando Normal strain. The orange-body and black-body nymphs responded similarly to fipronil- and indoxacarb-containing residues. Surprisingly, however, black-body nymphs exhibited significantly delayed mortality relative to orange-body nymphs on hydramethylnon-containing excretions from adult males. Reasons for this disparity, including the possibility that orange-body nymphs consume more feces than black-body nymphs, will be investigated in future research.

Nevertheless, the differences between orange- and black-body nymphs are minor compared to the differences between orange-body nymphs and PR-712 nymphs. In all instances we examined, mortality of Orlando Normal orange-body nymphs was significantly faster and greater than that of unselected and selected PR-712. Although this pattern is largely attributable to multi-resistance of PR-712 nymphs to fipronil, indoxacarb and hydramethylnon, we cannot rule out that the two strains differed in their consumption of the AI-containing adult excretions or their post-ingestive processing.

Commercial baits and AIs can also vary in secondary kill characteristics. Translocation of hydramethylnon and indoxacarb largely occurs via coprophagy,[8,10] whereas fipronil acts secondarily via emetophagy and contact.[12] In our study, all adults were removed before the nymphs were introduced, so horizontal translocation of AI through mechanisms other than coprophagy was most likely very limited, though surface contact of nymphs with toxic excretions was possible. Metabolic differences and differences in pre-ingestive sensory preferences of nymphs could also alter the lethality of excretions to nymphs. It is possible that coprophagy is more pronounced in laboratory colonies, and German cockroaches in the field (including PR-712) rely less on coprophagy, resulting in PR-712 nymphs ingesting less feces (AI) than Orlando Normal nymphs. Additionally, the two strains may differ in their qualitative preferences of adult feces. First instar nymphs offered equal amounts of adult male and female feces perform better (more likely to molt to the second stadium) on female feces.[15] Although no study has yet determined whether nymphs exhibit preferences for ingesting male vs. female feces, it is conceivable that differential preferences of the two strains contributed to differences in mortality.

We found that only ~70% of the insecticide susceptible nymphs died within 7 days on residues from PR-712 adults fed hydramethylnon bait (Figs 2F and I), whereas 100% of these nymphs died when exposed to residues from Orlando Normal adults fed the same bait (Fig. 2C). It is possible that PR-712 adults ingested less bait than Orlando Normal during the same time period, suggesting either lower general food intake, or a sensory avoidance of the bait. Glucose-averse cockroaches avoid glucose-containing baits,[30] and our preliminary evidence

suggests that a small fraction of the PR-712 population is sugar-averse,[31] possibly contributing to lower ingestion of baits. In addition, it is also possible that one of several mechanisms that underlie hydramethylnon resistance in PR-712 cockroaches is the catabolism and inactivation of hydramethylnon in the digestive tract. Both mechanisms would result in less AI in the adult feces, and lower mortality of nymphs exposed to adult feces. Nevertheless, the striking differences between the Orlando Normal and PR-712 nymphs, and especially of the artificially selected PR-712 line, support the conclusion that multi-AI resistance was the primary factor that significantly lessened secondary kill in PR-712 nymphs.

The horizontal transfer of AIs has potential advantages and disadvantages.[7] In the short-term, it results in secondary kill and presumably amplifies the direct effects of the AI, although it is important to note that all the evidence for these secondary effects come from laboratory and mesocosm studies and not from efficacy trials with field populations. On the other hand, we provide empirical support for the idea that translocation of AIs can expose low- or moderately resistant populations to sublethal doses of AIs, selecting for and causing a rapid increase in the frequency of resistance alleles, as discussed by Gressel (2010).[32] Coupled with other mechanisms that produce sublethal exposure to AIs in moderately resistant cockroaches (e.g., ingestion of less bait, glucose- and other nutrient aversions), exposure through contact and coprophagy to sublethal amounts of AI in conspecific feces may constitute an important mechanism that accelerates the development of insecticide resistance. This mechanism may counteract or even negate the advantages of secondary kill inherent to some bait products.

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Table 1. Toxicity, by topical application, of fipronil, indoxacarb, or hydramethylnon to adult males of a field-collected (PR-712) and a laboratory insecticide-susceptible strain of *Blattella germanica*.

Strain	Insecticide	*n*	Slope (±SE)	LD_{50} (95% CI)(µg·g^{-1})[a]	LD_{90} (95% CI)(µg·g^{-1})[b]	χ^2 (*d.f.*)	RR_{50}[c]	RR_{90}[d]
Orlando Normal	Fipronil	150	7.59 (±1.4)	0.04 (0.04-0.05)[e]	0.06 (0.05-0.07)[e]	1.999 (2)	–	–
PR-712 unselected	Fipronil	180	3.07 (±0.41)	0.22 (0.12-0.33)[f]	0.60 (0.39-1.81)[f]	9.749 (4)	5.6	9.78
PR-712 Fipronil selected	Fipronil	180	4.59 (±0.72)	0.64 (0.50-0.99)[g]	1.21 (0.84-4.61)[g]	4.491 (3)	15.93	20.2
Orlando Normal	Indoxacarb	330	6.21 (±0.91)	3.8 (3.2-4.5)[e]	6.1 (5-14.3)[e]	28.659 (8)	–	–
PR-712 unselected	Indoxacarb	210	0.9 (±0.12)	88.2 (9.3-323.2)[f]	2387.2 (559.7-442224.3)[f]	11.760 (4)	23.21	391.34
PR-712 Indoxacarb selected	Indoxacarb	210	0.66 (±0.22)	50839 (9318-134000000)[g]	~33317865 (147493.7-NA)[g]	3.008 (4)	13374.73	~54619.45[h]
Orlando Normal	Hydramethylnon	180	2.82 (±0.3)	19 (14.2-24.8)[e]	54.3 (39.8-85.9)[e]	6.830 (6)	–	–
PR-712 unselected	Hydramethylnon	210	1.59 (±0.19)	74 (23.7-186.2)[f]	474.7 (188.2-5512.5)[f]	11.099 (4)	3.9	8.74
PR-712 Hydramethylnon selected	Hydramethylnon	180	0.75 (±0.16)	367 (186.4-793.2)[g]	19054 (4961.9-422880)[g]	1.989 (3)	19.32	350.9

[ab] LD_{50}[a],LD_{90}[b] values with 95% confidence intervals. Values in µg active ingredient g^{-1} insect.

[cd] Resistance ratios at LD_{50}[c] and LD_{90}[d]. RR_{50} = LD_{50} value of PR712 strain / LD_{50} value of Orlando Normal strain.

[efg]Significant based on non-overlap of 95% confidence intervals among strains within insecticide applied.

[h]LD_{90} value cannot be accurately determined due to high-level resistance.

Strain weights: Orlando Normal 52.95 mg male^{-1}, PR-712 unselected 54.27 mg male^{-1}, PR-712 fipronil selected 53.72 mg male^{-1}, PR-712 indoxacarb selected 51.90 mg male^{-1}, PR-712 hydramethylnon selected 56.82 mg male^{-1}.

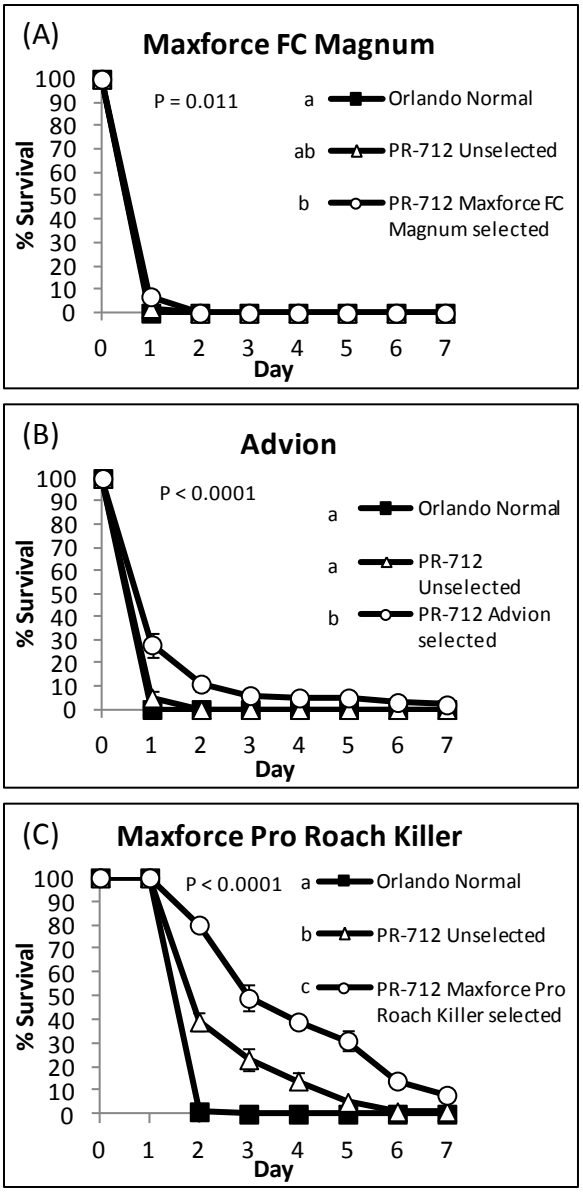

Figure 1. Survival of adult male *B. germanica* on three commercial bait products. (A) Maxforce FC Magnum cockroach bait (0.05% fipronil); (B) Advion cockroach bait (0.6% indoxacarb); (C) Maxforce pro roach killer (2.15% hydramethylnon). *P*–values determined with Log-rank test, plus-Sidak adjustment. *P*-values represent overall differences among strains. Strains not sharing lower case letter (adjacent to legend) are significantly different from each other.

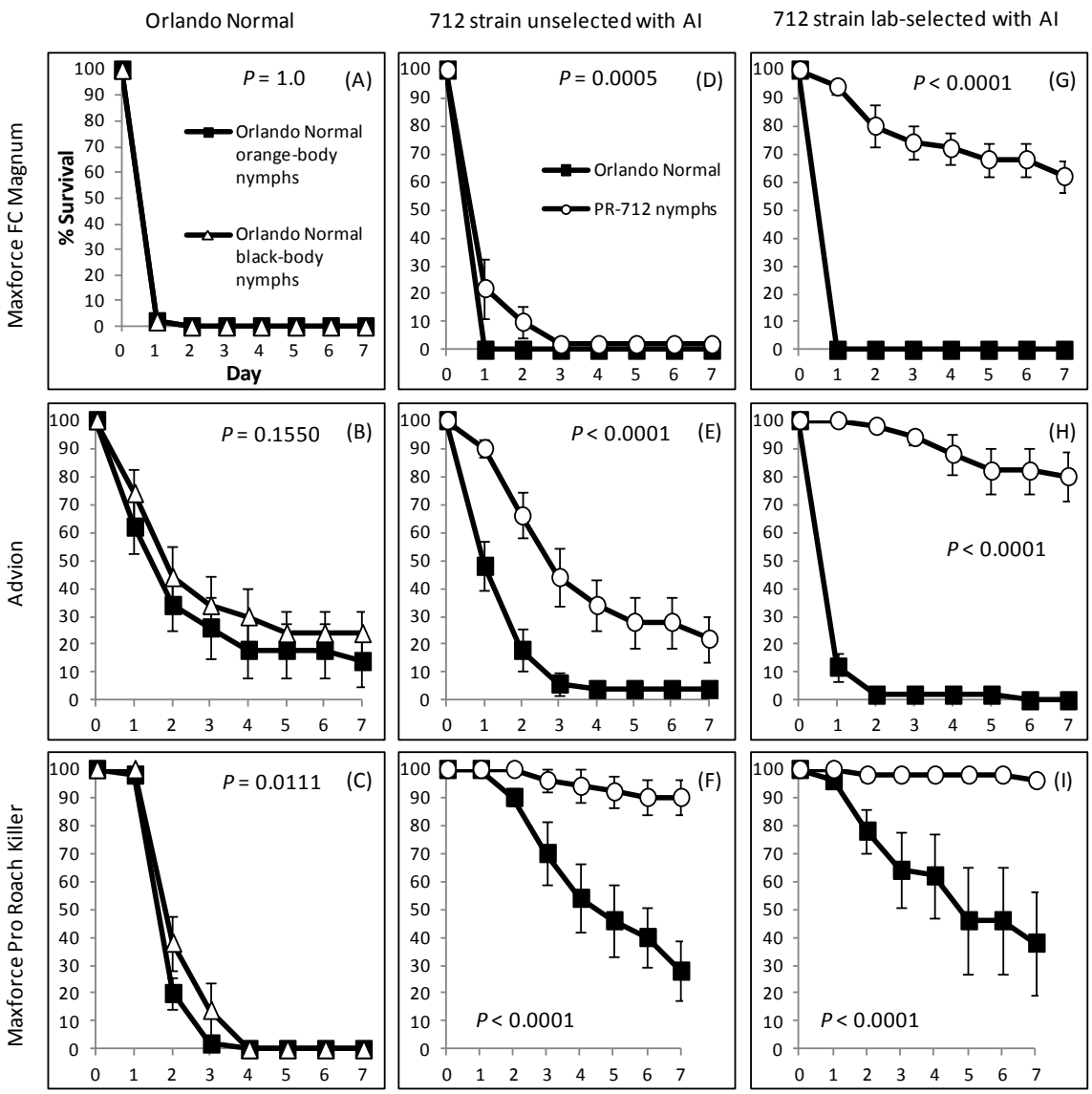

Figure 2. Survival of Orlando normal and PR-712 nymphs continuously exposed to excreta from Orlando normal or PR-712 males that were fed one of three commercial baits. (A-C) Survival of Orlando normal black-body and orange-body nymphs on excreta from black-body Orlando normal adult males. (D-F) Survival of Orlando normal orange-body nymphs and unselected PR-712 nymphs on the excreta of unselected PR-712 adult males. (G-I) Survival of Orlando normal orange-body nymphs and bait selected PR-712 nymphs on the excreta of selected PR-712 adult males. *P*–values determined with Log-rank test.

CHAPTER 2.

Diet quality affects bait performance in German cockroaches (Dictyoptera: Blattellidae)

(This work was submitted to the Journal of Pest Management Science)

Abstract

BACKGROUND:

Bait formulations are widely used to control German cockroach (*Blattella germanica*) populations. To perform optimally, these formulations must compete favorably with non-toxic alternative foods present within the insect's habitat. We hypothesized that the nutritional history of cockroaches and their behavioral ecology would affect their food preference and thus bait efficacy. To test this hypothesis, we conducted a controlled laboratory experiment, first giving glucose-accepting (Orlando Normal) and glucose-averse (T164) cockroaches nutritionally defined diets and then providing them with identical diets containing the insecticide hydramethylnon. Because the identity of non-active ingredients of commercial baits is proprietary, we used toxic diets as a bait proxy to evaluate the effect of diets of differing macronutrient composition on bait performance.

RESULTS:

The interaction between diet composition and bait composition affected the survival of adult males as well as first instar nymphs exposed to excretions produced by these males. Survival analyses indicated different responses of glucose-averse and glucose-accepting insects: Mortality of Orlando Normal males was highest when pre-exposed to high protein diets, whereas mortality of glucose-averse males was highest when pre-exposed to glucose containing diets, regardless of protein content. Secondary kill was also affected by the diet and bait combinations to which the adults were exposed. Any combination of diet and bait that resulted in high diet intake and low bait intake decreased secondary kill.

CONCLUSIONS:

We suggest that disparities between the nutritional quality of baits and the foods that are naturally available could have profound consequences in the management of German cockroach infestations. This study is the most comprehensive examination on the effect of alternative foods on bait efficacy.

KEY WORDS *Blattella germanica*, hydramethylnon, coprophagy, secondary kill, diet, bait

1 INTRODUCTION

The German cockroach, *Blattella germanica*, is a widespread pest of human-built structures. Several proteins produced by this cockroach can trigger allergic and asthmatic episodes[1-3] and *B. germanica* is a potential vector of pathogenic and antibiotic resistant microorganisms.[4-7]

Bait formulations are currently the most effective method of controlling German cockroach populations.[8,9] These are toxic diets that contain an active ingredient (AI) incorporated within a food matrix, which generally contains a sugar as a phagostimulant. In contrast to other methods of control, such as broadcast sprays, baits offer a more targeted approach resulting in less AI required for control and less AI exposure to non-target organisms such as children and pets.[8-10] A number of effective, non-repellent, active ingredients have been incorporated within baits.[8,9,11-15] Modern bait formulations can kill cockroaches through direct ingestion (primary kill), as well as through the uptake of translocated AI (secondary kill), whereby cockroaches, primarily 1st instar cockroach nymphs are adversely affected following ingestion of (coprophagy) or contact with excretions containing the toxicant.[12,15-19] Secondary kill through coprophagy is especially effective with slow acting AIs, such as hydramethylnon.[12,13]

While bait formulations are effective, multiple cockroach populations have evolved a chemosensory-based behavioral resistance to baits; in response to

selection with glucose-containing bait formulations, some cockroaches have evolved a taste aversion to glucose.[20] Although glucose is a common sugar and virtually universal phagostimulant, glucose-averse *B. germanica* reject bait formulations with glucose, surviving pest control efforts.[21,22]

In addition, for bait formulations to effectively control German cockroach populations, these toxic nutritive formulations must be preferred over other non-toxic alternative foods, and previous work has emphasized the importance of sanitation and the removal of alternative food sources.[8-10,23-25] The nutrient composition of diets can affect acceptance in this omnivore,[26-30] as prolonged exposure to suboptimal diets can result in specific nutrient deficiencies and physiological stress,[31-34] increasing the efficacy of palatable baits that satisfy these deficiencies.[10] *B. germanica* self-select optimal diets based upon their nutritional needs, reaching diet mixtures of approximately a 1:3 protein to carbohydrate ratio.[27-30] They also compensate for low dietary protein levels by elevating consumption rates, but extremely high dietary proteins can suppress food intake.[35]

We hypothesized that the interactions between the nutrient milieu available to the insects and their acceptance/avoidance of glucose would affect the efficacy of baits of various nutritional compositions. To test this hypothesis we exposed glucose-accepting and glucose-averse cockroaches to diets of varying protein to carbohydrate ratios, then introduced hydramethylnon-amended diets (hereafter termed “baits”) of similar or different macronutrient composition and recorded the

mortality of insects that ingested baits and those secondarily exposed to their excretions. We also hypothesized that glucose-averse cockroaches would reject diets containing glucose and thus suffer high mortality when subsequently exposed to palatable fructose-containing baits.

2 Experimental Methods

2.1 Stock insect colonies and rearing conditions

The strains of *B. germanica* used in these studies were Orlando normal and T164. Orlando normal is a glucose-accepting and insecticide-susceptible strain with no known prior exposure to insecticides; T164 glucose-averse cockroaches were collected from a Florida apartment in 1991.[21] Glucose aversion has been maintained in T164 by periodic laboratory selection with bait containing 11.8% glucose and 2% hydramethylnon. Neither strain has been reported to be resistant to hydramethylnon, the active ingredient used in this study. Colonies were maintained on Purina 5001 Rodent Diet (PMI Nutrition International, St. Louis, MO, USA) prior to the start of the study in laboratory rearing conditions of 25°C (± 1°C), 37 ± 5% RH and LD 12:12.

2.2 Characterization of susceptibility of strains to hydramethylnon

Hydramethylnon was serially diluted with acetone and 0.5 µL of a dilution was applied to the ventral surface of the cockroach, between the coxae of the fore- and middle-legs, with a repeating micro-pipette (Hamilton Company, Reno, NV, USA).

Thirty individuals were treated with each dose. Following treatment, cockroaches were maintained in three groups of 10 in 10 cm diameter petri dishes (Fisher Scientific, Pittsburgh, PA) with rat chow and water. Cockroaches topically treated with hydramethylnon were monitored for mortality daily for five days. Insects that could not right themselves within 30 sec when flipped were considered dead and removed from the petri dish. Values for LD_{50} and LD_{90}, and their respective fiducial limits, were estimated from Probit analysis in Polo Plus (LeOra Software Company, Petaluma, CA, USA).[36]

2.3 Composition and preparation of diets and baits

Diets were modified from Raubenheimer and Jones,[28] and ingredients are listed in Table 1. The digestible carbohydrate was either glucose or fructose. To prepare the diets, all dry ingredients were blended with 150 mL of dH_2O. Separately, agar was heated in 150 mL dH_2O until boiling. The agar mixture was allowed to cool after boiling to avoid denaturing the protein while continuously agitated to prevent congealing, before adding the other ingredients. Oleic acid dissolved in isopropanol was mixed into the agar solution, which was then mixed with the dry ingredient solution, and blended until homogeneous. To create the baits, hydramethylnon (CAS 67485-29-4, obtained from Bayer Corporation, RTP, NC) was dissolved with oleic acid in isopropanol and added to the nutrient solution, as described above. Diets and baits were poured into petri plates (100 mm x 15 mm), allowed to cool at room temperature for several hrs, stored at -20°C for 24 hrs, and then freeze-dried for five days, which also removed the isopropanol.

2.4 Effect of protein:carbohydrate (P:C) ratio and sugar type on diet consumption and lipid content of strains

We evaluated the effect of P:C ratio and sugar type on diet consumption and body lipid content to determine acceptability and carbohydrate assimilation of the treatment diets in both strains. We starved newly eclosed *B. germanica* males of the Orlando Normal and T164 strains for 24 hrs, with water provided, and then added one of four diets (P:C 1:3 with fructose; 1:3 with glucose; 3:1 with fructose; 3:1 with glucose) for three days. We used 15 replicates for each treatment. Each male had access only to a single treatment after eclosion, so its body lipid content was a direct consequence of the diet it consumed. To measure diet consumption, we cut the freeze-dried diets into cubes (approximately 0.125 cm^3), dried the diets for one week at 50°C, cooled in a desiccator, and weighed to the nearest 10 µg (Sartorius Model 1712 MP8). After diets had been exposed to the insects for the three-day consumption period, they were dried for an additional week at 50°C, cooled in a desiccator, and weighed again. Diet consumption was calculated as the difference in dry mass of the diets before and after insect feeding.

After we offered one of four diets for three days, each insect was dried for one week at 50°C, weighed, and then immersed for one week in 10 mL anhydrous diethyl ether in a 20 mL scintillation vial, with periodic agitation to extract lipids. After lipid extraction, diethyl ether was removed from the vials, and insects were dried at 50°C for an additional week, and then weighed. Lipid content was calculated as the

difference between dry mass of insects before and after extraction. To control for differences in starting dry mass between the two strains, 20 control insects of each strain were dried prior to the start of the experiment (mean ± SEM, Orlando Normal = 14.32 ± 0.295, T164 = 12.67 ± 0.342) and percentage lipid content was normalized to dry body mass.

2.5 Effect of diet and bait composition on male *B. germanica* mortality

To determine the effect of diet composition on bait efficacy, we exposed Orlando Normal and T164 adult males to P:C 1:3 or 3:1 diets, with the carbohydrate being either glucose or fructose. Males were deprived of food but provided with water for 24 hrs before introducing the diets. Diets were provided for three days, and then baits were added, allowing cockroaches access to both. There were no untreated controls in this experiment. We performed five replicates with 10 males per replicate. Mortality was recorded and dead insects were removed daily for 17 days. At the end of the experiment, the remaining insects were removed from the jars so that only adult excreta remained. 50 adults were used per treatment (1600 total).

2.6 Effect of diet/bait interactions on the toxicity of cockroach excretions

We predicted that excretions produced by male cockroaches exposed to different diet-bait combinations would vary in their availability, palatability and nutritional content, and thus would have different effects on secondary mortality of nymphs. We exposed newly emerged glucose-accepting orange-body 1st instar

nymphs (Orlando normal) and glucose-averse (T164) 1st instar nymphs to the residues produced by adult male *B. germanica* exposed to various diet and bait combinations, and recorded nymphal mortality. Ten first instar nymphs of each strain were confined to the same jar with the residues. The Orlando Normal nymphs were distinguished from T164 nymphs by body color using orange variants within the Orlando Normal colony.[37] Nymphs were first exposed to adult male excretions for 24 hrs, and then rat chow was added to the same jar. Five replicates per treatment were performed. Nymphal mortality was recorded and dead nymphs were removed daily for 10 days. Approximately 50 nymphs were used per treatment (1167 total), but several nymphs had escaped during the course of the experiment and were not included in the analysis.

2.7 Statistical analysis

Susceptibility of the two strains to hydramethylnon was statistically compared using the lethal dose ratio test, whereby tested strains are significantly different if the upper and lower 95% confidence intervals do not encompass 1.[36] The effect of diet on percent lipid content per dry body mass was analyzed with ANOVA and post-hoc Tukey's HSD for multiple comparisons. Diet consumption and lipid content analysis was implemented in R (v 3.1.2). One T164 adult escaped, thus only 59 adults were used for T164 compared to 60 Orlando Normal adults. For both primary and secondary kill experiments, treatments were compared with Kaplan-Meier survival analysis and log-rank tests. All survival analyses were implemented in SAS 9.3

(SAS, Cary, NC). A Sidak adjustment was used for the log-rank test multiple comparisons.

3 RESULTS AND DISCUSSION

3.1 Susceptibility of Orlando Normal and T164 to hydramethylnon

Topical application of 0.5 µL acetone did not cause any mortality. The LD_{50} values (95% CI) of Orlando Normal and T164 were 21.33 (15.93 - 27.81, χ^2 = 6.8094) and 37.90 (34.68 - 41.90, χ^2 = 3.9430) $\mu g \cdot g^{-1}$, respectively. Likewise, the LD_{90} values (95% CI) of Orlando Normal and T164 were 60.75 (44.62 - 95.92) and 61.97 (53.99 - 76.12) $\mu g \cdot g^{-1}$, respectively. The LD_{50} and LD_{90} resistance ratios (RR) of T164 relative to Orlando Normal were 1.78 and 1.02, respectively. While the LD_{50} values of the two strains were significantly different (Lethal dose ratio test; 95% CI, 0.449 – 0.705), the LD_{90}'s were not (0.710 – 1.355). Orlando Normal males weighed significantly more at eclosion [47.33 ± 0.871 mg dry mass (± SEM)] than T164 males (42.84 ± 0.654; T-test: $t = 4.114_{35.3}$, $P = 0.00022$).

3.2 Effect of protein:carbohydrate ratio and sugar type on diet consumption and lipid content

Whole-body lipids generally increase as sugar and fat intake increases.[27,29] Varying the dietary P:C ratio and the incorporated sugar significantly affected the amount of diet consumed by the two strains of cockroaches (Fig. 1). Orlando Normal males fed high-carbohydrate diets containing fructose or glucose

consumed more food than on high-protein diets, and as expected, their lipid contents were highest (Fig. 1). Generally, consumption of P:C 3:1 diets was lower than 1:3 diets, as expected, and Orlando Normal males on these diet treatments significantly differed in the conversion of food to body lipids. Orlando Normal males consuming high carbohydrate diets of either sugar contained more lipids than males given high protein diets (ANOVA: $F_7 = 11.372$, $P < 0.0001$; Fig. 1), resulting in a positive correlation between diet intake and body lipids (Spearman correlation: S = 23394, n = 60, $r_S = 0.3450$, $P = 0.0061$).

T164 glucose-averse insects largely rejected glucose-containing diets and consumed significantly greater amounts of fructose-containing diets, regardless of their P:C ratio. T164 males also consumed less carbohydrate-rich diet than Orlando Normal males, even when the sugar offered was fructose (Fig. 1). The pattern of diet consumption and consequent lipid accumulation differed in T164 males. Males provided P:C 1:3 or 3:1 fructose-containing diets accumulated lipids in a similar pattern as Orlando Normal males, with more body lipids on sugar-rich diets. However, T164 males offered glucose-containing diets exhibited an unusual reversed relationship between intake and lipid accumulation. Males fed high protein (3:1) glucose diet ate more but accumulated less lipids than males fed 1:3 glucose diet. Thus, males were able to extract more lipid precursors from the unpalatable 1:3 glucose diet than from the more palatable but non-preferred high P diet. In contrast to the positive correlation between diet intake and body lipid content in Orlando Normal males, no overall correlation was found in T164 males when all four

diets were considered (Spearman correlation: S = 31728, n = 59, r_S = 0.0728, P = 0.5837).

These results, suggesting that T164 insects may have different metabolic/nutritional requirements from Orlando Normal, are consistent with those of Shik *et al*.[29] and Jensen *et al*.,[30] who also reported lower fructose diet intake of T164 than Orlando Normal insects. However, whereas Shik *et al*.[29] found similar lipid content in T164 and Orlando Normal nymphs fed 1:3 fructose diets, we found less lipid in T164 males (Fig. 1). It is likely that differences in experimental insects (nymphs vs. adults), their physiological condition (premolt vs. postmolt), and experimental conditions (number of days in the experiment) contributed to this divergence of results.

These results add to a growing body of evidence that evolutionary shifts in diet breadth of cockroaches[20,29,30,38,39] can have downstream effects on life history characteristics such as sexual maturation[40] and courtship.[41] We suggest that glucose-averse cockroaches have evolved lower carbohydrate needs, an adaptation that may facilitate the persistence of glucose-aversion in German cockroach populations in the absence of selection with glucose-containing baits.

3.3 Effect of diet and bait composition on male *B. germanica* mortality

Mortality of Orlando Normal males was affected by the P:C ratios of both their diet and the hydramethylnon bait. Three days of feeding on a suboptimal

P:C 3:1 diet improved the efficacy of all bait combinations tested (Figs 2b and d), while feeding on optimal 1:3 diets reduced bait performance, regardless of the type of sugar used (Figs 2a and c). Greater separation of survival curves was observed on the 1:3 diets, revealing preferences of Orlando Normal insects for high carbohydrate baits regardless of sugar type, and for high protein (3:1) glucose baits over high protein fructose baits (Figs 2a and c). Nevertheless, optimal 1:3 baits of either sugar performed well regardless of the composition of the pretreatment diets. We suggest that even slight differences in sugar preferences may have significant effects on bait performance.

With T164 males, fructose-containing baits always performed the best (Figs 2e,f,g and h; Table 2). While many T164 glucose-averse males survived treatments with glucose-containing baits, nearly all survived when first provided with 1:3 fructose diets (Fig. 2g). Baits containing fructose were most effective against T164 when insects were first exposed to non-preferred diets (1:3 glucose, 3:1 glucose or 3:1 fructose) (Figs 2e,f and h). Thus, satiating cockroaches with optimal diets that match intake targets (1:3 fructose) rendered both glucose baits and high-protein fructose baits ineffective, and decreased the effectiveness of 1:3 fructose baits by 60% (Fig. 2g). This result further illustrates that the macronutrient composition of food and satiety state of insects can dramatically affect bait choice in glucose-averse insects.

Despite aversion to glucose by T164 males, glucose-containing baits still killed insects that had fed a low-quality or non-preferred diet for 3 days (Figs 2e,f and h). These findings suggest that males that were essentially deprived of food for 3 days (high glucose diets dramatically suppress feeding in T164 males – Fig. 1) accept some glucose bait. It is also possible that persistent exposure to glucose in petri dishes caused some sensory adaptation to glucose-containing foods.[42] Regardless, aversion to glucose by T164 insects trumps macronutrient composition.[29]

3.4 Effect of diet/bait interactions on the toxicity of cockroach excretions

First instar *B. germanica* nymphs ingest the feces of conspecifics, an adaptive behavior whereby these relatively sedentary nymphs procure critical nutrients for development.[12,16-18] The macronutrient composition of feces produced by adult *B. germanica* largely mirrors that of their diet.[18] We demonstrated that nymphs were profoundly affected by the macronutrient compositions of the diet and bait offered to adult males. As with male mortality, nymphal mortality was also a function of sugar type, P:C ratio, and strain. Specifically, any diet and bait combination that lowered adult diet intake and promoted bait intake (and hence hydramethylnon excretion) also elevated secondary kill of nymphs.

For example, nymphs of both strains suffered high mortality (~80%) when exposed to feces produced by males fed a high-protein diet (i.e., lower intake) and high-fructose bait (i.e., higher intake) (Fig. 3c). Glucose-averse T164 males fed a

high-glucose diet (i.e., lower intake) and then exposed to a more palatable fructose-containing high protein bait also resulted in higher secondary kill of nymphs (Fig. 3b). T164 males presumably avoided the unpalatable diet in favor of the fructose-containing bait, even though its P:C ratio was suboptimal, and thus they excreted more hydramethylnon-laden feces.

Conversely, any diet and bait combination that increased adult diet intake and decreased bait intake also decreased secondary kill of nymphs. For example, offering Orlando Normal males a combination of a preferred diet (high-glucose or high-fructose) and a non-preferred bait (high-protein) produced low secondary nymphal mortality (Figs 3a,b and e). Feeding either Orlando Normal or T164 males high-fructose diet (P:C 1:3 fructose) resulted in little secondary mortality of nymphs with 3:1 bait (Fig. 3e), presumably because little bait was consumed and excreted. Similarly, few Orlando Normal and T164 nymphs died on the excretions of adult Orlando Normal males that had been fed 1:3 glucose diet followed by 3:1 fructose bait (Fig. 3b), presumably because Orlando Normal adults preferred the optimal 1:3 diet over the 3:1 suboptimal bait, resulting in limited excretion of hydramethylnon in feces. Low adult mortality in the Orlando Normal strain (Fig. 2a, red line) given this diet and bait combination reflect these results. Finally, low nymphal mortality occurred when male cockroaches of either strain were fed a P:C 1:3 glucose diet, followed by a 3:1 glucose bait (Fig. 3a), presumably because of two different mechanisms. Orlando Normal males were likely satiated and thus consumed little of the 3:1 bait, whereas T164 males likely avoided both the diet and bait because they

contained glucose; both strains would produce little feces after bait exposure and cause little secondary mortality.

Comparisons of Figure 2d and 2h illustrate how differences in sensory mechanisms can affect both primary and secondary kill. Moderate mortality was evident in T164 adults provided P:C 3:1 fructose diet followed by 1:3 glucose bait (Fig. 2h, green line); likely due to low intake of the 1:3 glucose bait, little nymphal mortality was observed in both strains on the adult T164 residues (Fig. 3f, red and blue lines), a probable consequence of limited hydramethylnon in the adult excretions. However, most Orlando Normal males fed this same diet and bait combination died, likely due to a preference of the 1:3 glucose bait over 3:1 fructose diet (Fig. 2d, green line). When exposed to the excretions produced by these males most Orlando Normal nymphs died while most T164 nymphs survived (Fig. 3f). Because there is no substantial difference in the susceptibility of the two strains to hydramethylnon, we propose that T164 nymphs survived because they avoided glucose in the excreted feces. While previous studies have demonstrated positive correlation between ingested and excreted carbohydrates in the feces of German cockroaches[18] and locusts,[43] the presence of specific monosaccharides that reflect dietary intake will need to await confirmation through direct analysis.

Comparisons of adult male Orlando Normal mortality when offered P:C 1:3 fructose or 3:1 fructose diet followed by 1:3 glucose bait (Figs 2c and 2d), with Orlando Normal nymphal mortality (Figs 3d and 3f) revealed an interesting insight

about the role of nutrition in secondary kill. When Orlando Normal adults were given 3:1 fructose diet, then given 1:3 glucose bait, mortality occurred very quickly, and all insects died by day 6, three days after bait introduction (Fig. 2d, green line). However, when Orlando Normal adults were given 1:3 fructose diet, rather than 3:1 fructose diet, and then given 1:3 glucose bait, mortality occurred more slowly, resulting in only 60% mortality of adults by day 6 (Fig. 2c, green line). In the first scenario, with Orlando Normal adults fed 3:1 fructose diet, and then 1:3 glucose bait, their excretions killed 90% of the Orlando Normal nymphs (Fig. 3f, green line). However, in the second scenario when adults were fed a 1:3 fructose diet, then 1:3 glucose bait, only 33% of Orlando Normal nymphs died on the adult excretions (Fig. 3d, green line). Thus, the secondary kill properties of bait formulations are considerably affected by available alternative foods, the palatability of food and bait, and insecticide resistance,[44] which affect the ingestion of bait by foraging insects, their survival, and the amount of feces they deliver to aggregation sites.

These results highlight that both primary and secondary kill performance of highly preferred baits can be compromised by the availability of equally preferred alternative foods through three mechanisms: (1) Adults eat less of the bait because they had become satiated on the alternative preferred diet, (2) Adults produce less toxicant-laden feces, and (3) The toxicant becomes diluted in feces produced from the optimal non-toxic diet. The differential prominence of these mechanisms will depend upon the array of foods available, the physiological stage of adults and nymphs and their life stages. For example, Kopanic *et al*.[18] demonstrated that

development of first instars was better supported by adult female feces than by equal amounts of male feces, suggesting that the nutritional quality of male and female feces may differ.

3.5 THE ROLE OF ALTERNATIVE FOODS ON BAIT PERFORMANCE AND INSECTICIDE RESISTANCE

Alternative food sources are thought to interfere with cockroach control, and their removal (i.e., improved sanitation) has been promoted as a key component of effective cockroach control, especially with baits.[8-10,23-25] However, no studies have examined how the relative qualities and acceptability of these foods and baits affect pest control. In this study we examined the effect of various foods on bait performance in a controlled laboratory environment where food and bait consumption, lipid content and mortality (primary and secondary kill) could be quantified. It is important to note, however, that under normal operational conditions in the field, and with commercial baits, the consumption of baits will be substantially guided by olfactory cues (attraction) and gustatory cues (palatability and phagostimulation). Olfactory cues can improve baiting success by luring German cockroaches over long distances[45,46] and phagostimulants can bias the insects' consumption, independent of nutritional needs;[45] indeed, commercial baits rank differently on attractiveness[46] and palatability scales.[45] Moreover, in many insects, responses to olfactory[47] and gustatory[48] cues are heightened when either starved or deprived of key nutrients. Field *B. germanica* appear unable to meet their macronutrient intake target,[33] suggesting that nutritionally balanced baits with

effective attractants and phagostimulants should be highly effective in these nutritionally austere environments.

The effects of palatable baits can be magnified through secondary kill, whereby the bait is ingested and translocated by foraging cockroaches, excreted within or near cockroach aggregations, and the AI is ingested again by non-foraging members of the population. The magnitude of secondary kill can be modulated by many factors, but primary among these is the type of AI and amount of bait ingested.[13,45] Our results show that both nutritional and sensory mechanisms can reduce the interaction of nymphal cockroaches with adult feces. The effects of sensory mechanisms on the interaction of cockroach nymphs with adult feces are most readily seen in T164 nymphs; these nymphs ingested less glucose-containing feces, thus not only reducing the efficacy of baits, but also exposing cockroaches to sublethal doses of AI. Insecticide resistance has also been shown to result in sublethal doses of AI delivered to coprophagous nymphs,[44] suggesting that the presence of alternative foods may influence the development of resistance in a similar manner.[49]

Lastly, numerous studies have linked cockroach nutritional status[50,51] and fat body accumulation[52] with insecticide resistance through greater capacity to metabolize xenobiotics,[53] suggesting that alternative foods might play a role in insecticide resistance beyond simply influencing the palatability of commercial baits.

4 SUMMARY AND CONCLUSIONS

Bait efficacy is influenced by many factors, including its attractiveness and palatability,[45,46] toxicity of different active ingredients,[14,15,45] presence of resistance alleles within the target insect population,[44,54-59] its ability to affect secondary mortality,[12-14] and practical considerations such as bait distribution.[60] Here we demonstrate that bait performance could also be affected by a predictable interaction between the nutritional condition of the German cockroach, which is a consequence of its nutritional history, and bait macronutrient composition. The relative quality and palatability of the bait, in relation to the previous food consumed, affect bait intake and primary mortality, as well as AI excretion and secondary mortality.

The mortality profiles we observed reflected the *B. germanica* macronutrient intake target of 1:3 protein:carbohydrate reported by Jones and Raubenheimer,[27] Raubenheimer and Jones,[28] Shik, *et al*.,[29] and Jensen *et al*.,[30] lending further support to a close and predictable relationship between diet and bait intake levels and mortality. Baits were most effective when they matched the intake target and were preceded by food that departed from the intake target. Conversely, baits became increasingly ineffective when cockroaches fed on high quality food before offered the bait. These principles readily extended to glucose-averse cockroaches, with the added constraint that glucose transformed nutritionally adequate diets and baits (i.e., P:C 1:3) into poor-quality analogs. Thus, baits that corresponded to the intake target performed poorly on T164 if they contained glucose.

Moreover, we demonstrated that the toxicity of male excretions to nymphs (secondary kill) varied with cockroach strain, diet and bait P:C ratio, and sugar type. The combination of high diet intake and low bait intake resulted in less secondary kill, whereas the combination of low diet intake and high bait intake resulted in greater secondary kill. The complexity of this diet-bait macronutrient interaction was further extended when glucose-averse *B. germanica* adults were offered diets and baits with and without glucose. Here, intake of diet and/or bait was a function of the glucose-aversion trait, and foods that would normally be preferred (1:3) were rejected. Thus, glucose aversion had the potential to both increase acceptance of diets (if baits contained glucose) or increase bait efficacy (if diets contained glucose). Consequently, the survival of nymphs exposed to the excretions was the result of the macronutrient interactions of the diet and bait the adults were given.

Sensory mechanisms (olfaction and taste) and insecticide resistance are two factors that play prominent roles in the efficacy of cockroach baits. This study, and a related investigation,[44] have shown that the efficacy of baits can be compromised, resulting in sublethal doses of AI to foraging cockroaches and coprophagous nymphs, potentially hastening the evolution of insecticide resistance.[49]

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Table 1. Compositions of *B. germanica* diets and baits

Ingredients[a]	P:C 1:3[b]	P:C 3:1[b]
Casein	7.50	22.50
Peptone	3.75	11.25
Albumin	3.75	11.25
Carbohydrate	45.00	15.00
Cellulose	26.90	26.90
Agar	4.00	4.00
Vitamin mixture	0.81	0.81
Hydramethylnon	1.00	1.00
2-propanol	6 ml	6 ml
Oleic acid	1 ml	1 ml

[a]Ingredients comprising diets (no hydramethylnon) and baits (with hydramethylnon). The carbohydrate source was either glucose or fructose. Ingredients were obtained from the following suppliers: Casein – Sigma Aldrich, St Louis, MO (C5890); peptone – BDH (440754K); albumin – BDH, Poole, UK (Cat. No. 830083G; alpha-cellulose – Sigma (C8002); agar – Oxoid, Basingstoke, UK (L11); Hydramethylnon (Bayer corporation); Vanderzant vitamin mix – Sigma (V1007).

[b]P:C 1:3 and 3:1 indicate protein:carbohydrate ratios. Unless noted otherwise, compositions of ingredients are in grams.

Table 2. Statistical comparisons of primary (male) mortality for all diet-bait combinations shown in Figure 2

Strain	Diet type	Comparison of bait types[a]		DF	Chi-square	Sidak adjusted P-value
Orlando Normal	P:C 1:3 glucose		GLOBAL	3	171.13	<0.0001
		PC 3:1 fructose	PC 3:1 glucose		25.12	<0.0001
		PC 3:1 fructose	PC 1:3 fructose		119.00	<0.0001
		PC 3:1 fructose	PC 1:3 glucose		116.90	<0.0001
		PC 3:1 glucose	PC 1:3 fructose		18.60	<0.0001
		PC 3:1 glucose	PC 1:3 glucose		17.65	0.0002
		PC 1:3 fructose	PC 1:3 glucose		0.02	1.0000
	P:C 3:1 glucose		GLOBAL	3	47.76	<0.0001
		PC 3:1 fructose	PC 3:1 glucose		3.12	0.3826
		PC 3:1 fructose	PC 1:3 glucose		12.41	0.0026
		PC 3:1 fructose	PC 1:3 fructose		43.00	<0.0001
		PC 3:1 glucose	PC 1:3 glucose		2.96	0.4154
		PC 3:1 glucose	PC 1:3 fructose		21.86	<0.0001
		PC 1:3 glucose	PC 1:3 fructose		8.50	0.0211
	P:C 1:3 fructose		GLOBAL	3	123.89	<0.0001
		PC 3:1 fructose	PC 3:1 glucose		7.96	0.0284
		PC 3:1 fructose	PC 1:3 glucose		71.69	<0.0001
		PC 3:1 fructose	PC 1:3 fructose		79.89	<0.0001
		PC 3:1 glucose	PC 1:3 glucose		24.90	<0.0001
		PC 3:1 glucose	PC 1:3 fructose		28.93	<0.0001
		PC 1:3 glucose	PC 1:3 fructose		0.17	0.9989
	P:C 3:1 fructose		GLOBAL	3	38.55	<0.0001
		PC 3:1 fructose	PC 3:1 glucose		0.00	1.0000
		PC 3:1 fructose	PC 1:3 glucose		18.47	0.0001
		PC 3:1 fructose	PC 1:3 fructose		18.52	0.0001
		PC 3:1 glucose	PC 1:3 glucose		18.36	0.0001
		PC 3:1 glucose	PC 1:3 fructose		18.34	0.0001
		PC 1:3 glucose	PC 1:3 fructose		0.00	1.0000
T164	P:C 1:3 glucose		GLOBAL	3	107.96	<0.0001
		PC 1:3 glucose	PC 3:1 glucose		0.02	1.0000
		PC 1:3 glucose	PC 1:3 fructose		42.82	<0.0001
		PC 1:3 glucose	PC 3:1 fructose		38.27	<0.0001
		PC 3:1 glucose	PC 1:3 fructose		41.61	<0.0001
		PC 3:1 glucose	PC 3:1 fructose		37.12	<0.0001
		PC 1:3 fructose	PC 3:1 fructose		0.10	0.9997
	P:C 3:1 glucose		GLOBAL	3	68.44	<0.0001
		PC 1:3 glucose	PC 3:1 glucose		1.01	0.8958
		PC 1:3 glucose	PC 1:3 fructose		37.71	<0.0001
		PC 1:3 glucose	PC 3:1 fructose		33.24	<0.0001
		PC 3:1 glucose	PC 1:3 fructose		23.35	<0.0001
		PC 3:1 glucose	PC 3:1 fructose		20.02	<0.0001
		PC 1:3 fructose	PC 3:1 fructose		0.12	0.9996
	P:C 1:3 fructose		GLOBAL	3	213.47	<0.0001
		PC 1:3 glucose	PC 3:1 glucose		0.43	0.9867
		PC 1:3 glucose	PC 3:1 fructose		30.97	<0.0001
		PC 1:3 glucose	PC 1:3 fructose		123.40	<0.0001
		PC 3:1 glucose	PC 3:1 fructose		23.43	<0.0001
		PC 3:1 glucose	PC 1:3 fructose		104.40	<0.0001
		PC 3:1 fructose	PC 1:3 fructose		21.43	<0.0001
	P:C 3:1 fructose		GLOBAL	3	87.64	<0.0001
		PC 3:1 glucose	PC 1:3 glucose		0.93	0.9144
		PC 3:1 glucose	PC 3:1 fructose		35.10	<0.0001
		PC 3:1 glucose	PC 1:3 fructose		41.62	<0.0001
		PC 1:3 glucose	PC 3:1 fructose		21.67	<0.0001
		PC 1:3 glucose	PC 1:3 fructose		26.70	<0.0001
		PC 3:1 fructose	PC 1:3 fructose		0.28	0.9958

[a]Pairwise comparisons (6) of the four survival curves within a given graph represented in Figure 2. Global indicates overall differences among all four survival curves within a graph.

Table 3. Statistical comparisons of the nymphal secondary mortality curves shown in Figure 3

Diet [a]	Bait [a]	Adult strain [b]	Nymphs exposed [b]	compared to:	Adult strain [c]	Nymphs exposed [c]	DF	Chi-square	Sidak adjusted *P*-value [d]
				GLOBAL			3	7.79	0.0506
P:C 1:3 glucose	P:C 3:1 glucose	Orlando	T164		T164	T164		0.01	1.0000
		Orlando	T164		T164	Orlando		2.36	0.5497
		Orlando	T164		Orlando	Orlando		4.82	0.1574
		T164	T164		T164	Orlando		2.62	0.4882
		T164	T164		Orlando	Orlando		5.17	0.1300
		T164	Orlando		Orlando	Orlando		0.43	0.9862
				GLOBAL			3	62.81	<0.0001
P:C 1:3 glucose	P:C 3:1 fructose	Orlando	Orlando		Orlando	T164		0.04	1.0000
		Orlando	Orlando		T164	T164		31.08	<0.0001
		Orlando	Orlando		T164	Orlando		32.66	<0.0001
		Orlando	T164		T164	T164		27.93	<0.0001
		Orlando	T164		T164	Orlando		29.41	<0.0001
		T164	T164		T164	Orlando		0.02	1.0000
				GLOBAL			3	1.61	0.6567
P:C 3:1 glucose	P:C 1:3 fructose	T164	Orlando		T164	T164		0.01	1.0000
		T164	Orlando		Orlando	Orlando		0.47	0.9828
		T164	Orlando		Orlando	T164		1.15	0.8648
		T164	T164		Orlando	Orlando		0.40	0.9890
		T164	T164		Orlando	T164		1.06	0.8854
		Orlando	Orlando		Orlando	T164		0.16	0.9990
				GLOBAL			3	10.30	0.0162
P:C 1:3 fructose	P:C 1:3 glucose	T164	T164		T164	Orlando		2.40	0.5406
		T164	T164		Orlando	T164		7.88	0.0296
		T164	T164		Orlando	Orlando		7.70	0.0327
		T164	Orlando		Orlando	T164		1.54	0.7648
		T164	Orlando		Orlando	Orlando		1.33	0.8196
		Orlando	T164		Orlando	Orlando		0.02	1.0000
				GLOBAL			3	1.06	0.7871
P:C 1:3 fructose	P:C 3:1 glucose	T164	T164		Orlando	T164		0.01	1.0000
		T164	T164		T164	Orlando		0.78	0.9411
		T164	T164		Orlando	Orlando		0.34	0.9929
		Orlando	T164		T164	Orlando		0.64	0.9629
		Orlando	T164		Orlando	Orlando		0.25	0.9970
		T164	Orlando		Orlando	Orlando		0.10	0.9998
				GLOBAL			3	133.00	<0.0001
P:C 3:1 fructose	P:C 1:3 glucose	T164	T164		T164	Orlando		0.64	0.9639
		T164	T164		Orlando	T164		2.31	0.5630
		T164	T164		Orlando	Orlando		86.56	<0.0001
		T164	Orlando		Orlando	T164		0.55	0.9747
		T164	Orlando		Orlando	Orlando		73.51	<0.0001
		Orlando	T164		Orlando	Orlando		58.88	<0.0001

[a]Diet and bait combinations given to the adult males.
[b,c]Survival of nymphs[b] exposed to residues from adults[b] of a given strain compared to another survival curve of nymphs[c] given residues from adults[c].
[d]All pairwise comparisons (6) are shown within each group, with the corresponding significance values. Significance of the global comparisons indicate differences among all four survival curves within a given group represented in Figure 3.

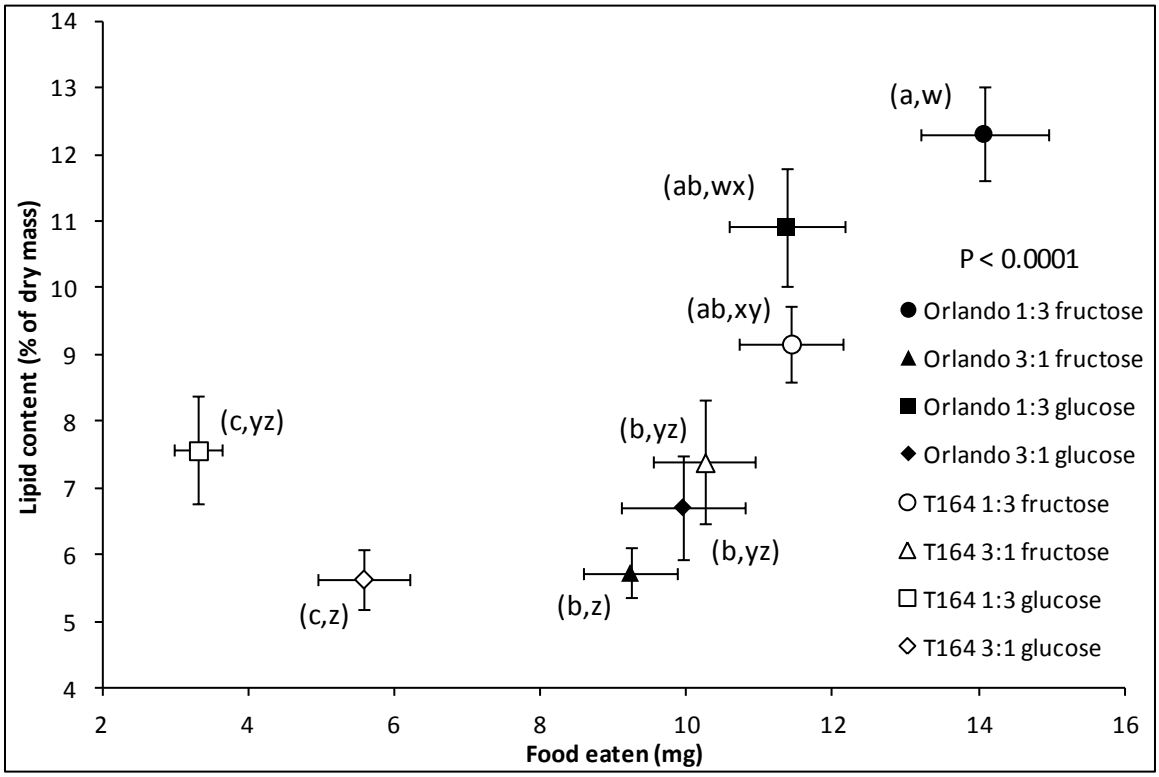

Figure 1. Relationship between dry mass of food eaten (mg) and lipid content (percent of dry body mass) for Orlando Normal (closed symbols) and T164 (open symbols) adult males. Ratios (1:3 and 3:1) represent protein:carbohydrate ratios. Points with different letters in parentheses are significantly different from each other with respect to their axes [food eaten (a,b,c,d), lipid content (w,x,y,z)] (one-way ANOVA, post-hoc Tukey test for multiple comparisons). Each mean (± SEM) represents 15 replicates for diet intake and 15 replicates for lipid content.

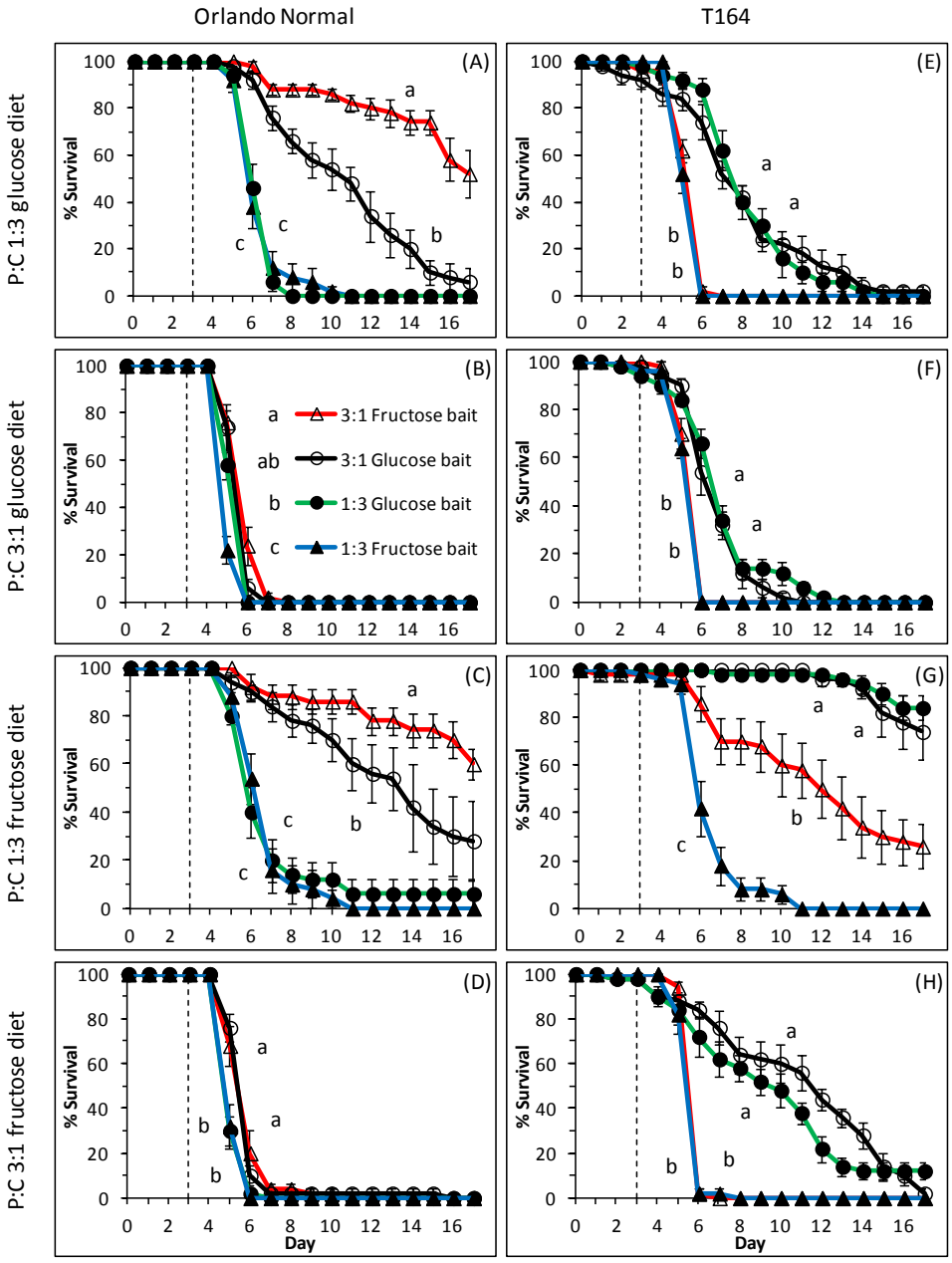

Figure 2. Effects of two bait monosaccharides and protein:carbohydrate (P:C) ratios on adult male survival in relation to their feeding history on various diets. Orlando Normal and T164 males were fed one of four different diets (noted on left) for three days followed by one of four baits on day 3, represented by dotted vertical line. SEM shown for each mean, and statistical analyses are shown in Table 2. 50 insects were used per treatment (1600 total).

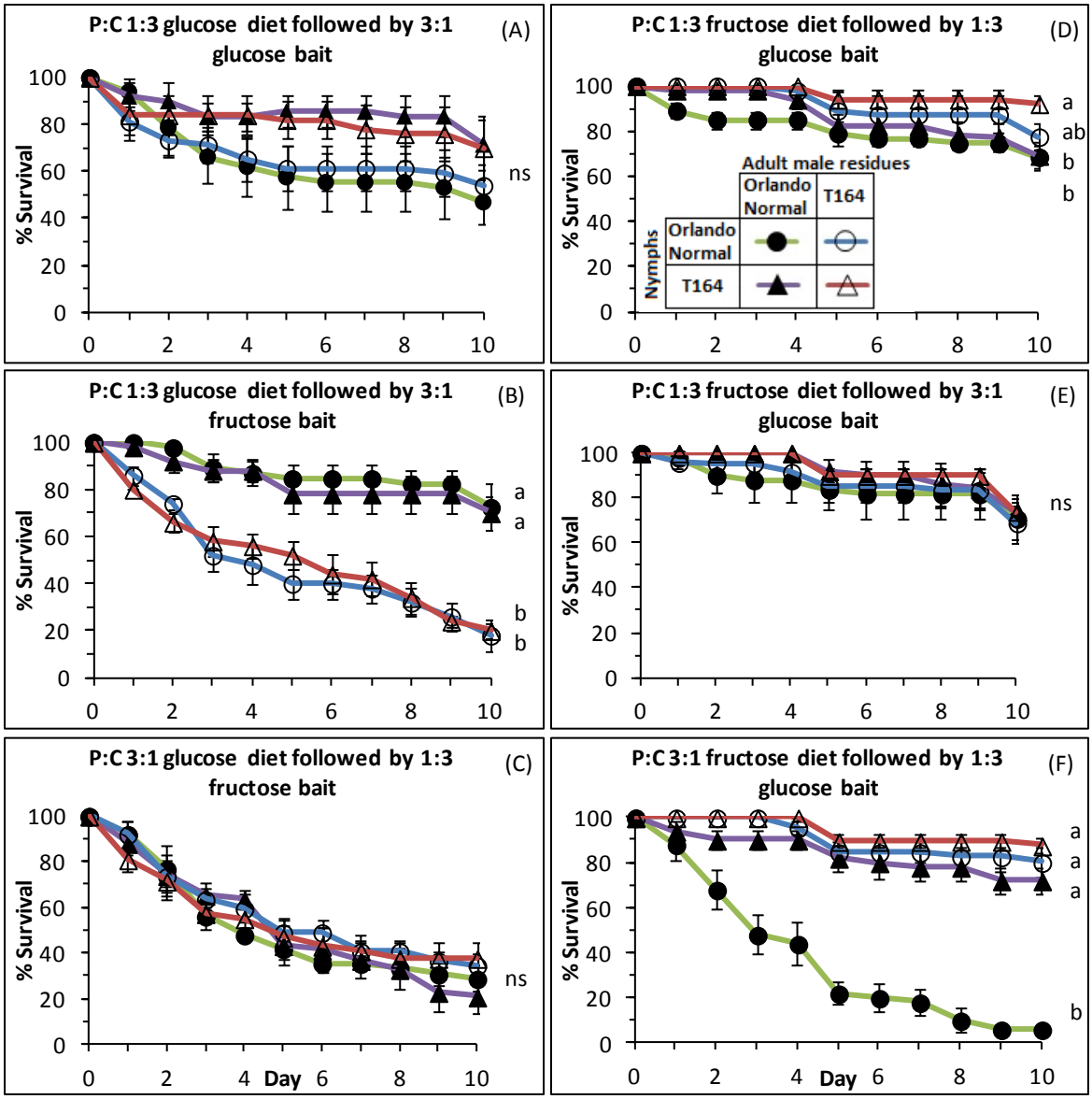

Figure 3. Mortality of 1st instar nymphs on residues left by adult male *B. germanica* fed various diets and exposed to various baits. Ratios (P:C 1:3 and 3:1) represent protein:carbohydrate ratios. SEM shown for each mean, and statistical analyses are shown in Table 3. Survival lines with different letters indicate significant differences, with "ns" representing non-significant differences among lines. Approximately 50 nymphs of each strain were used per treatment (1167 total).

CHAPTER 3.

Insecticide resistance and nutrition interactively shape life-history parameters in the German cockroach, *Blattella germanica*

(This work has been submitted to Scientific Reports)

ABSTRACT

Fitness-related costs of evolving insecticide resistance have been reported in a number of insect species, but the interplay between evolutionary adaptation to insecticide pressure and variable environmental conditions has received little attention. We compared developmental parameters of three German cockroach (*Blattella germanica* L.) populations differing in insecticide resistance, under optimal and marginal nutritional conditions. A field-collected strain was upon collection separated into two populations, which were maintained either without insecticide exposure or with bimonthly exposure to indoxacarb in bait formulation for 2.5 years. Nymphs were, since hatching, provisioned with either nutritionally rich (ground rat chow) or poor (ground rat chow diluted 1:1 with cellulose) diet during development. Our results show that indoxacarb resistance and poor nutritional condition both increased development time to the adult stage and lowered adult body size and body mass upon eclosion, with reinforcing interactions. We also found lower survival to the adult stage in the indoxacarb-selected population, which was exacerbated on poor diet. In addition, nutrition imparted a highly significant effect on cockroach susceptibility to indoxacarb. Our study exemplifies how poor nutritional condition can aggravate the life-history costs of resistance and elevate the detrimental effects of insecticide exposure, demonstrating how environmental conditions may impact insecticide efficacy.

INTRODUCTION

Insecticide resistance, a heritable adaptation to insecticide pressure, provides a valuable system to study the evolutionary ecology of adaptation to severe environmental hazards and the consequences to fitness-related life-history parameters within the adapted population. The evolution of insecticide resistance is often accompanied by lower fitness of resistant populations under insecticide-free conditions compared to susceptible populations[1-11]. Moreover, both the magnitude of resistance and the frequency of resistant individuals often decline rapidly in the absence of selection pressure[12-15], indicating that the maintenance of resistance incurs direct performance costs, most likely through higher metabolic rate and re-allocation of resources from somatic growth and reproduction to sustain elevated resistance mechanisms[4,16,17].

Environmental conditions including the nutritional quality of available food impact physiological body condition which in turn affects performance[18-20]. The quality of the available diet may furthermore interact with heritable life-history strategies and genetic adaptations to environmental hazards[21,22]. For example, diets that support good body condition typically also support higher immune response and survival upon pathogen exposure[18,23], and animals in good body condition would similarly be expected to better resist and detoxify secondary metabolites and insecticides. Although it is well known that nutrient availability may play an important role in the ability of insects to handle and detoxify xenobiotics[24-27], the interactive effects of nutritional condition and insecticide resistance on life-history outcomes have to our knowledge not been investigated.

The German cockroach (*Blattella germanica* L.) is an obligate pest in the anthropogenic environment and populations have been targeted with a multitude of control measures, most prominently including various insecticides. In response, German cockroach populations have evolved an array of physiological and behavioral mechanisms of insecticide resistance[28-33]. An early study on life-history costs associated with resistance to DDT showed longer development time and smaller adult body mass in a population selected for resistance compared to the original unselected population[34], and resistant field-collected strains showed similar patterns when compared to a susceptible laboratory strain[35]. Subsequent experiments provided further support for life-history related costs associated with insecticide resistance in German cockroaches[1,34-37]. However, a recent study could not confirm this association when comparing resistant field-collected strains to a susceptible laboratory strain after 5 years of laboratory rearing without insecticide exposure[38].

We used recently collected German cockroaches with moderately high resistance to indoxacarb to investigate interactive effects between nutritional condition and resistance to insecticide on life-history characteristics. Indoxacarb functions as a sodium channel blocker upon metabolic transformation into toxicologically active metabolites[39]. It is formulated into baits for cockroach control, but recent reports have documented decreased performance of indoxacarb bait due to evolution of resistance in German cockroach populations[40,41]. In diamondback moths (*Plutella xylostella* L.), resistance to indoxacarb has been associated with higher production of esterase enzymes that presumably break down the active

components[42,43] and sodium channel mutations that reduce sensitivity to indoxacarb[44]. Similarly, esterases are implicated in indoxacarb resistance in maize weevils (*Sitophilus zeamais* M.)[45], but multiple mechanisms appear to operate in house flies (*Musca domestica* L.) including limited activation of the pro-insecticide[46]. In the German cockroach, biochemical inactivation likely plays a prominent role in indoxacarb resistance, but only sodium channel mutations are well-documented[47]. Life-history costs to evolving indoxacarb resistance have been reported only in moths (*Heliothis virescens* F. and *Spodoptera exigua* H.)[6,48] and a homopteran (*Phenacoccus solenopsis* T.)[15].

We compared performance parameters among three German cockroach populations with different levels of indoxacarb resistance raised under rich and poor nutritional conditions. We also investigated survival of moderately resistant cockroaches exposed to indoxacarb after completing development on rich or poor diet. We hypothesized that the cost of adaptation to the insecticide would be more evident under poor nutritional conditions, and that insecticide resistance and poor nutrition would interactively elevate fitness costs. We furthermore hypothesized that nutritional condition alone would significantly affect the susceptibility of cockroaches to insecticide exposure.

METHODS

Cockroach populations

Insecticide-resistant and -susceptible strains of German cockroaches originated from two field-collected populations. The insecticide susceptible strain (Orlando Normal; ON) was collected more than 60 years ago in Orlando, Florida and has since collection been maintained as a standard, unselected laboratory population with no resistance to any insecticides[41,49]. Insecticide-resistant cockroaches (PR-712) were collected in Carolina, Puerto Rico in August 2012, at a location where multiple insecticides failed to control the population[41]. Upon collection, this population was separated equally into two laboratory-maintained populations – one that was selected with indoxacarb over 2.5 years, and one that was maintained unselected with no exposure to insecticide. We selected for indoxacarb resistance by provisioning Advion gel bait (Syngenta, Greensboro, NC, U.S.A., containing 0.6% indoxacarb) over three consecutive days every two months. This regimen selected cockroaches approximately every generation. After 2.5 years of recurrent artificial selection, the three populations showed highly distinct levels of resistance to indoxacarb matching their adaptive backgrounds[41]. All populations were maintained at high density in transparent plastic containers (46 cm × 23 cm × 30 cm high) with ad libitum access to water and rat chow (Purina 5001 Rodent Diet; PMI Nutrition International, St. Louis, MO, U.S.A.). Experiments and rearing were performed in a room at 25±2ºC and a 12:12 h L:D regime.

Diets

We produced diets with either high or low nutrient content based on ground and sieved rat chow. Diet of high nutrient content (rich diet) consisted of rat chow in 4% agar, and diet of low nutrient content (poor diet) consisted of rat chow diluted 1:1 by mass with crystalline α-cellulose (Sigma C-6429, Sigma-Aldrich, St. Louis, MO, U.S.A.), also in 4% agar. The ground rat chow was sieved through a 0.71 mm mesh. Crystalline cellulose served as a bulking agent and has very limited nutritional value. Both diets were mixed in water at 50°C after boiling the water with the agar, and freeze-dried. Diets were provided in biscuit-like cubes of up to 1 cm^3 and resupplied as needed to ensure ad libitum availability.

Effects of diet on life-history parameters

Nymphs from each of the three populations were allocated to transparent glass jars (10 cm diameter × 10 cm height) in groups of 10 nymphs per jar within 24 h of hatching (n = 30 jars per population and diet). The jars contained a piece of egg carton for harborage and were covered with paper towel squares held in place with rubber bands. The inner walls of the jars were lined with a thin layer of petroleum jelly and mineral oil mixture to prevent climbing. Nymphs had free access to water provided in cotton-plugged glass tubes (9 mm diameter × 75 mm length) that were continuously replaced. Newly eclosed adults were collected and recorded within 24 h of emergence. Individual body mass upon eclosion was determined by weighing to the nearest mg and pronotum width was measured with a calibrated eyepiece micrometer in a dissecting microscope.

Effect of diet on susceptibility to indoxacarb

Upon eclosion to the adult stage, males from the unselected PR-712 population were maintained individually in Petri dishes (100 mm diameter × 16 mm height) with a water tube and ad libitum access to the same diet as during development. At eight days post-emergence, the males were briefly anesthetized with carbon dioxide, and 50 µg of indoxacarb in 0.5 µL of acetone solution was applied topically on the ventral side of the thorax. This dose was selected based on a preliminary experiment where we topically applied 50, 100, or 150 µg indoxacarb to adult males collected from the culture. Individuals were checked daily for survival and recorded as dead (or dying) when unable to stand on their legs. Maximum survival was set at 28 days, which substantially exceeded the period of indoxacarb lethality. We chose to test the effect of dietary condition on susceptibility in this population because we expected this population to best represent the original field population.

Statistical analyses

Overall effects of population, diet, and sex on survival to the adult stage, development time, adult pronotum width, and mass upon eclosion were analyzed using proportional hazard tests (JMP 7.0, SAS Institute Inc., Cary, NC, U.S.A.), and multiple comparisons across populations, diets, and sexes were conducted using Kruskal-Wallis tests at a significance level of $p < 0.05$ (SPSS 22.0, IBM Institute Inc., Chicago, IL, U.S.A.). Survival of males from the unselected PR-712 population after

indoxacarb exposure was compared between dietary treatments using a Log-Rank test (SAS 9.4, SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Survival to the adult stage

We found significant effects of both population and diet on survival to the adult stage (Proportional hazard; population: $\chi^2_{2,180} = 8.7538, p = 0.0126$; diet: $\chi^2_{1,180} = 7.3995, p = 0.0065$). Whereas the majority of nymphs survived to the adult stage across all populations and diets, survival was lower in the indoxacarb-selected population than in the other populations and significantly lower when developing on nutrient-poor diet (Fig. 1).

Development time

Both population and diet significantly influenced development time to the adult stage (Table 1, Fig. 2a). On rich diet, development time was fastest in the ON population for both sexes, significantly longer in the unselected PR-712 population, and even more protracted in the indoxacarb-selected population (Fig. 2a). Development was significantly longer on nutrient-poor diet in both sexes within all populations (Table 1, Fig. 2a). We found a significant interaction between population and diet on development time (Table 1), indicating that development time was further prolonged by poor diet in the indoxacarb-selected population than in the other two populations

(Fig. 2a). We also found a significant interaction between population and sex on development time (Table 1), which might reflect longer median development time of males than females in the indoxacarb-selected population, whereas females had longer median development time in the other populations (Fig. 2a).

Adult body size

Pronotum width of emerging adults differed significantly depending on both population and diet (Table 1, Fig. 2b). Adult ON females were larger than females from both of the other populations with significant differences on nutrient-rich diet (Fig. 2b). In contrast, female cockroaches from the indoxacarb-selected population that had developed on nutrient-poor diet were significantly smaller than the females in all other population and diet groups (Fig. 2b). Similar patterns were apparent for the males (Fig. 2b). We found a significant interaction between population and diet on adult body size (Table 1), reflecting that body size was significantly affected by nutrition within both sexes in the indoxacarb-selected population but less affected by nutritional condition within the other two populations (Fig. 2b). We also found a significant interaction between population and sex on body size (Table 1), which might reflect overall larger median size differences between populations within females than within males (Fig. 2b).

Adult body mass

Body mass upon eclosion similarly differed significantly depending on both population and diet (Table 1, Fig. 2c). Adult ON females had the largest median

body mass upon eclosion (Fig. 2c). In contrast, indoxacarb-selected adult females had significantly smaller median body mass upon eclosion than females from the other populations within both diets (Fig. 2c). Male body mass upon eclosion was similar across populations and diets in the ON and PR-712 unselected populations, but significantly smaller in the indoxacarb-selected population reared on poor diet (Fig. 2c). We found a significant interaction between population and diet on body mass (Table 1), indicating a larger effect of diet in the indoxacarb-selected population than in the other populations (Fig. 2c). We also found a significant interaction between population and sex on body size (Table 1), which likely reflects gradual differences in female body mass across the three populations whereas male body mass was more constant across populations (Fig. 2c). We furthermore found a significant interaction between population, diet, and sex (Table 1). This likely reflects effects of both population and diet on female body mass, whereas poor diet only compromised body mass in males from the indoxacarb-selected population (Fig. 2c).

Dietary effect on susceptibility to indoxacarb

Survival upon topical application of 50, 100, or 150 µg indoxacarb to adult males from the PR-712 strain varied depending on dose and population (Fig. 3). We selected the 50 µg dose to test for effects of nutritional condition in PR-712 unselected males because it caused larger variation in survival than higher doses (Fig. 3). Susceptibility of PR-712 unselected males to indoxacarb was significantly higher under poor than under rich nutritional condition (Log-Rank: $\chi^2_{1,256} = 50.53, p < 0.0001$; Fig. 4). The majority of males (62%) given nutrient-rich diet survived 50 µg of

indoxacarb (Fig. 4). In contrast, 50% of the males restricted to nutrient-poor diet died within three days of indoxacarb exposure and 75% were dead before 10 days. Only 22% of the males given nutrient-poor diet survived the indoxacarb treatment (Fig. 4).

DISCUSSION

Insecticides impose high selection pressure on populations to evolve resistance mechanisms to circumvent the hazard, and modify life-history traits as a trade-off to alleviate the costs of resistance[1,2,6,8-11]. However, the life-history responses of insects to the potentially complex interactions of insecticide resistance with environmental factors such as dietary conditions have been little investigated. We found interactive costs of evolving high indoxacarb resistance and developing under poor nutrition on several life history traits, including time to reach the adult stage and adult body measures (size and mass), in German cockroach populations (Table 1, Fig. 2). We also found a significant influence of nutritional condition on survival after direct exposure to insecticide (Fig. 4).

Survival to first reproduction is arguably the most important determinant of individual fitness. Therefore, absolute survival to the adult stage is not expected to trade-off dramatically with other life-history parameters, while the time under development and final size and mass may vary. Our finding that survival to the adult stage was significantly lower in the highly resistant population raised on poor diet indicates that the evolution of high resistance to indoxacarb is associated with severe costs, and that some individuals are not able to compensate for these costs,

especially under poor nutritional conditions (Fig. 1). Significantly longer development times in the highly resistant population under both rich and poor nutritional conditions further indicate severe costs associated with evolving resistance to indoxacarb (Table 1, Fig. 2a), and the very long development time in this population under poor nutritional condition shows that these nymphs struggled to gain enough resources for development and growth. Nevertheless, it is important to note that high insecticide resistance may impose costs on some life-history parameters and not on others. For example, selection with insecticides reduced fecundity and reproductive rate in some bed bug (*Cimex lectularius* L.) populations, but it did not diminish longevity and generation time was shorter in selected populations[10].

Although highly significant, insecticide resistance and diet had lower effects on adult body size and mass than on development time (Table 1, Fig. 2), such that adult size and mass was prioritized at the cost of prolonged development time. This prioritization was previously shown in *B. germanica*[50] and more extensively in the moth *Manduca sexta* L.[51,52], indicating that the fitness costs associated with small adult size and mass are higher than costs associated with longer development time. In female insects, fecundity typically increases with body size[53]. However, body size usually has little effect on mating success in females whereas smaller males are at a disadvantage in sexual selection due to lower performance under female choice and in male-male competition[54,55]. Maturing at a smaller adult size therefore means fewer chances to sire offspring in males while only the number of offspring produced per breeding cycle is likely to be affected in females. Larger size-dependent fitness consequences in males than in females thus likely explain the interactions between

population and sex on development time, body size, and mass, and between population, diet, and sex on adult body mass in our experiment (Table 1, Fig. 2).

Diet quality significantly affected the susceptibility of male German cockroaches to indoxacarb (Fig. 4). Various toxicokinetic processes could be affected by diet, including cuticle permeability and metabolic degradation of the insecticide. Resistance to indoxacarb is primarily mediated by detoxification and target-site mutations[42-47], and the availability and quality of nutritional resources are expected to impact the former through constitutive and up-regulated synthesis of detoxifying enzymes. Esterases, glutathione-*S*-transferases and cytochrome P450-dependent monooxygenases, which mediate biochemical resistance, are energetically costly to produce, require substantial investment in amino acids, and their synthesis also requires other nutritional components such as sulfur and iron[9,17]. Higher metabolic rate[4,16] and expenditures in amino acid and in limited micronutrients to sustain higher production of detoxification enzymes likely explain the higher dependence on rich nutritional conditions by the highly resistant cockroach population in our experiment, as resources are allocated to resistance mechanisms instead of growth. In this context, it would be interesting to determine whether target-site mutations that confer high resistance to insecticides reduce the metabolic investment in detoxification enzymes, which might uncouple the dependence on nutrition to maintain resistance. It could also be interesting to knock down the expression of detoxification genes, e.g. by RNA interference, and measure the effect on life-history parameters under variable conditions.

German cockroach populations have evolved behavioral resistance to ingesting insecticides by detecting and avoiding the active ingredient in bait[30], or by evolving specific aversions to certain bait components[56]. In cabbage loopers (*Trichoplusia ni* H.), performance costs associated with resistance to *Bacillus thuringiensis* (Bt) toxins were found to be connected to lower consumption by the resistant strain even though no toxin was present[22]. Similarly, indoxacarb-resistant cockroaches might have adapted to insecticide-containing baits by maintaining inherently lower consumption even in the absence of any insecticide. This would lower the risk of ingesting deleterious amounts of insecticide while facilitating the evolution of physiological resistance. If consumption is inherently low in highly resistant individuals, this might be part of the interactive effect between resistance and poor nutrition recorded in the present experiment, in addition to the likely effects of metabolic and resource allocation costs.

In the absence of insecticide, optimal growth can be quickly regained due to natural selection for lower resistance that alleviates the resistance costs[12,13,15], and this likely explains the lack of fitness penalty observed after rearing insecticide-resistant German cockroach populations in the laboratory with no insecticide pressure[38]. It is possible that after 2.5 years of laboratory rearing with no insecticide pressure and ad libitum feeding conditions, cockroaches in our PR-712 unselected population might have evolved higher developmental performance than upon collection. This population might therefore have adapted to life histories more similar to those of the ON population than when the culture was established, although individuals were still highly resistant when given rich nutrition (Fig. 4).

In the present study, males collected directly from the unselected PR-712 culture were intermediately susceptible to 50 µg indoxacarb (Fig. 3) compared to the unselected PR-712 males that had developed on either rich or poor diet in our experiment (Fig. 4). This exemplifies that also other environmental factors than diet influence individual condition and susceptibility to insecticide. Since our population cultures are maintained at relatively high density, stress factors such as pathogen exposure and competition are likely to be high in our cultures compared to the more isolated experimental conditions. In addition to direct effects of infection, higher infection risk cascades to up-regulated immune responses[57,58], which due to trade-offs may increase susceptibility to other environmental hazards[59]. Males taken from the culture were furthermore of unknown age and were therefore likely to be generally older than experimental males, which is likely to have further decreased their body condition.

In the field, nutritional conditions are expected to be suboptimal compared to laboratory conditions[60], especially if efforts are made to deplete the availability of potential food sources. Paradoxically, laboratory studies examining the effects of insecticides are typically performed under rich nutritional conditions. The present study underscores the substantial influence of nutritional condition on the costs of insecticide resistance on insect fitness parameters in the absence of insecticide, but also on individual tolerance to direct insecticide exposure. Our results emphasize that a key component of insecticide deployment strategies (especially when applying toxic bait) – minimizing the access to food sources – would be particularly effective in controlling populations that are resistant to insecticides.

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Conflict of interest

None of the authors has any financial or other interests concerning the outcomes of the investigation or any conflicts of interest to declare.

Author contributions

K.J. and A.E.K. carried out the study. K.J. analyzed the data. K.J., A.E.K., C.S., and J.S. wrote the manuscript.

Table 1. Proportional hazard tests on the effects of population (Orlando Normal, PR-712 unselected, or PR-712 indoxacarb-selected), diet (rich or poor), sex (male or female) and their interactions on development time to the adult stage, adult body size (pronotum width), and body mass upon eclosion.

Dependent variable	Factor	*df*	χ^2	*p*
Development time	Population	2	598.90	<0.0001
	Diet	1	455.61	<0.0001
	Sex	1	3.55	0.0596
	Population × diet	2	18.69	<0.0001
	Population × sex	2	11.44	0.0033
	Diet × sex	1	1.19	0.2759
	Population × diet × sex	2	2.34	0.3100
Body size	Population	2	147.61	<0.0001
	Diet	1	37.35	<0.0001
	Sex	1	1993.64	0.0000
	Population × diet	2	28.38	<0.0001
	Population × sex	2	9.04	0.0109
	Diet × sex	1	2.17	0.1405
	Population × diet × sex	2	0.66	0.7172
Body mass	Population	2	242.12	<0.0001
	Diet	1	19.67	<0.0001
	Sex	1	1689.00	0.0000
	Population × diet	2	16.80	0.0002
	Population × sex	2	11.31	0.0035
	Diet × sex	1	0.02	0.8867
	Population × diet × sex	2	14.09	0.0009

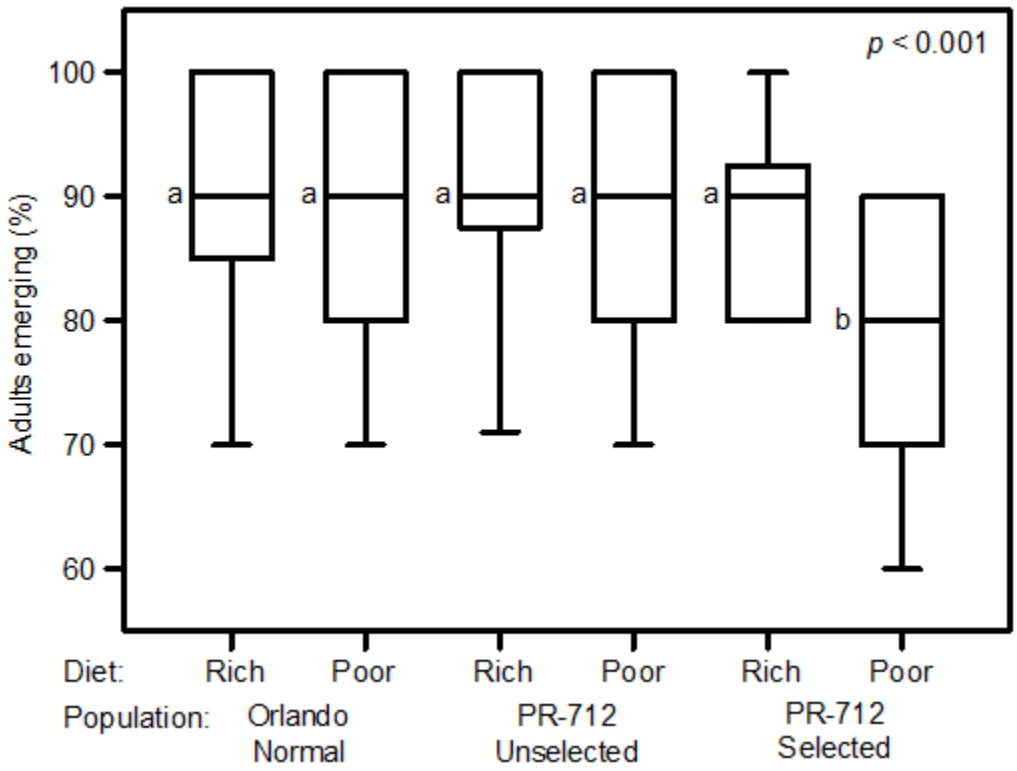

Figure 1. Percentage of individuals surviving development to the adult stage per jar for each cockroach population and dietary condition. Nymphs were set up in groups of 10 per jar within 24 h upon hatching. 30 jars were set up for each population and diet. Boxes show median ± 25th percentiles and 95% confidence intervals. The overall *p*-value is from a Kruskal-Wallis test. Different letters indicate significant differences among populations and treatments at a significance level of $p < 0.05$ (Kruskal-Wallis post-hoc test).

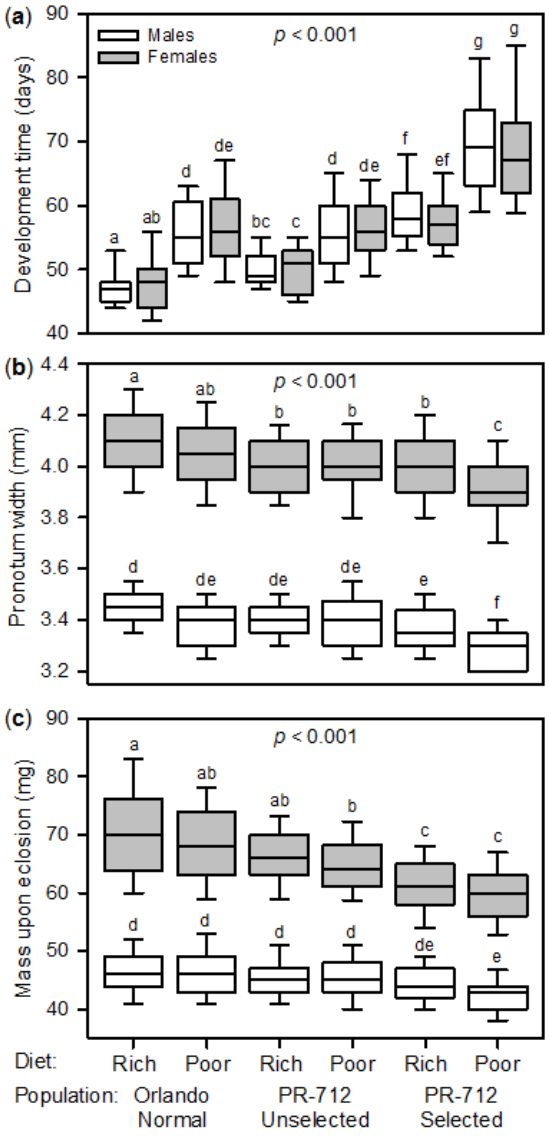

Figure 2. (a) Development time, (b) adult pronotum width, and (c) body mass upon eclosion in individuals surviving to the adult stage. Nymphs were set up in groups of 10 per jar within 24 h of hatching. 238 to 286 adults emerged per population and diet. Boxes show median ± 25th percentiles and 95% confidence intervals. The overall *p*-values are from Kruskal-Wallis tests. Different letters indicate significant differences among populations and treatments at a significance level of $p < 0.05$ (Kruskal-Wallis post-hoc tests).

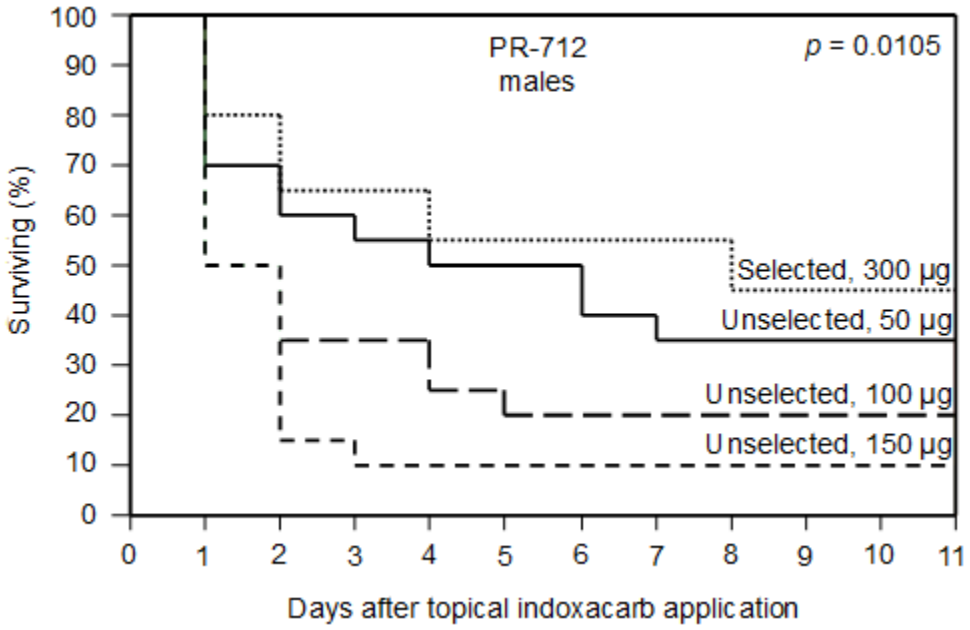

Figure 3. Survivorship curves for PR-712 males from the unselected population after topical application of 50, 100, or 150 µg indoxacarb per insect. We also included a treatment using males from the PR-712 indoxacarb-selected population that we topically treated with 300 µg indoxacarb. 20 adult males were used in all treatments. Males were taken directly from the population cultures before topical application and were maintained individually in 10 cm Petri dishes with a cotton-plugged water tube and ad libitum rat chow following application. Survival was recorded daily over 11 days. The *p*-value is from a Log-Rank test.

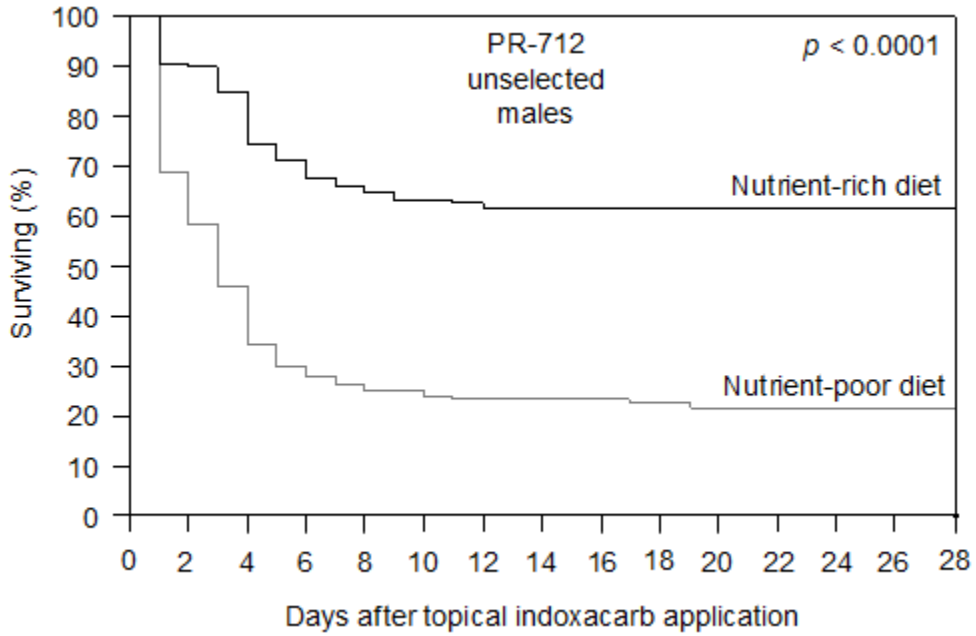

Figure 4. Survivorship curves for PR-712 males from the unselected population after topical application of 50 µg indoxacarb per individual. Insects were reared to adults in groups of 10 on nutritionally rich or poor diet since hatching, after which males were maintained individually in 10 cm Petri dishes with a cotton-plugged water tube and ad libitum access to their respective diet. Indoxacarb was applied on day 8 after adult emergence, and survival was recorded daily over 28 days. The *p*-value is from a Log-Rank test.

CHAPTER 4.

Greater foraging distance accelerates growth and reproduction in an insect omnivore

ABSTRACT

The German cockroach (*Blattella germanica*) is an excellent model omnivore for studying the effect of foraging distance on nutrient balancing behavior and physiology, and its consequences on performance. We investigated the effect of foraging distance on individual cockroaches by providing nutritionally complementary foods diverging in protein and digestible carbohydrate content at opposite ends of short and long foraging arenas. To balance their intakes of the two diets, cockroaches had to repeatedly traverse the length of the arena. After seven days of foraging, we determined specific nutrient consumption, mass growth, lipid accumulation, and oocyte growth as a measure of reproductive maturation. Contrary to predictions, nymphs in longer foraging arenas increased their protein intake relative to carbohydrate intake and had greater structural mass as a result. Furthermore, adult females foraging longer distances gained more weight and matured their oocytes faster than females foraging over short distance. Our study shows a positive effect of greater foraging on two different growth parameters via different adjustments to specific nutrient intake.

Keywords: *Blattella germanica*, carbohydrate, protein, foraging distance, nutrient balancing, performance.

INTRODUCTION

According to optimal foraging theory, foraging strategies and behaviors that benefit organismal fitness will tend to increase in frequency over time (Pyke 1984). A multitude of factors influence foraging behavior, and primary among them are extrinsic pressures, such as predation (density and distribution in time and space), food composition, intrinsic requirements and constraints including nutrient balancing, prior foraging experience, and the energetic cost of acquiring nutrients (Pyke 1984). A balanced diet is particularly critical because deficits in the amounts and proportions of specific nutrients could lead to nutritional stress and negative consequences for growth, development, sexual attractiveness and reproduction (Simpson et al. 1995; Simpson & Raubenheimer 2012). A wide range of animals have been shown to optimally balance their intake of nutrients (Simpson et al. 2004, 2015; Raubenheimer and Jones 2006; Lee, et al., 2008; Simpson and Raubenheimer 2012; Jensen et al. 2012, 2013).

However, to consume specific nutrients optimally, animals must often forage for, and consume mixtures of foods that may be separated in time or space. One may postulate that carbohydrates, a universal metabolic fuel, are required for foraging excursions between different foods, especially if singular foods are inadequate for proper development. Even walking, a less energetically taxing activity compared to running or flying, requires mobilization of carbohydrates and lipids (Lorenz & Gade 2009). Most of the support that carbohydrate is the main metabolic fuel during muscular exertion comes from literature on flight in bees

(Suarez et al. 2005). Thus it follows that as foods become more spatially separated, foraging becomes increasingly energetically taxing, and demands for digestible carbohydrate would increase.

However, studies on the effect of long distance foraging on specific nutrient consumption seem to contradict this expectation. Van der Zee et al. (2002) compared desert locust consumption of high protein and high carbohydrate foods at varying distances, and found that as the distance between the foods increased, insects remained near and consumed more high protein foods. Similarly, Behmer et al. (2003) found that when locusts were challenged with differently sized circular arenas containing high protein and high carbohydrate foods, they spent more time feeding on the side of the arena containing the protein food compared to the carbohydrate food. Behmer et al. (2003) also found that locusts grew at the same rate, regardless of arena size. Surprisingly, body lipid levels increased in locusts as arena size increased, not expected if locusts needed to respire ingested carbohydrates in order to forage in larger arenas. Both authors find that locusts exhibit greater food fidelity to high protein foods in large arenas, presumably due to inherent predator avoidance behavior. As distance between foods increases, exposure to predators also increases (Bernays et al. 1997), although chemically defended insects such as locusts may be less affected by food distance (Chambers et al. 1996). Although locusts can travel great distances, it is obvious that even small scale manipulations yielded changes in specific nutrient consumption (Van der zee et al. 2002; Behmer et al. 2003; Bernays et al. 1997).

Short-distance foraging allows insects to feed from foods more frequently and enables them to regulate their food consumption more tightly than long distance foraging. Of course, the level of energy expenditure required to forage depends upon the natural activity level of the organism. Organisms that exhibit higher activity levels than locusts could have greater carbohydrate demands, and may show a greater effect of distance on food consumption. Similarly, organisms that have no natural enemies may not show similar patterns of food fidelity, which has been attributed to predator avoidance (Behmer et al. 2003; Chambers et al. 1996).

The German cockroach (*Blattella germanica*) is a highly active omnivore that exists within human built structures despite hostile conditions and often sparse food supplies (Rust et al. 1995; Schal 2011). Adapted to an extremely transient and convoluted nutritional landscape, with patches that vary both in quantity and quality of resources (Rust, et al., 1995), German cockroach nymphs have a very high capacity to redress nutritional imbalances (Raubenheimer and Jones 2006). Furthermore, it has an impressive foraging range (Rivault and Cloarec 1991) and therefore has the potential to compose a nutritionally balanced diet from complementary food sources that are widely separated. Having no natural enemies (Rust et al. 1995), these natural foraging conditions make the German cockroach an ideal model system for testing feeding behavior of extremely mobile foragers in space, our goal being to explain the effect of foraging distance on nutrient balancing and subsequent performance outcomes.

Here, we conduct two experiments to understand the fitness consequences of foraging distance on German cockroach food consumption, growth, and reproduction. Logically, foraging between complementary nearby foods should be beneficial because of the ease with which insects can nutritionally balance them for an optimal diet. To examine this, we first confine both male and female newly eclosed last instar nymphs within long and short foraging arenas, and measure diet consumption and growth. Then we record the effect of foraging distance on oocyte length, a measure of reproductive maturation, in adult females. We hypothesize that as the distance between two nutritionally diverse food sources increases, consumption of carbohydrate will increase due to the elevated energy expended on foraging.

METHODS

(a) Experimental animals

The cockroaches used in our experiments originated from the Orlando Normal strain and were reared on rat chow (Purina 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO, USA). Rearing and experiments were performed at 25 ± 2 °C, 35 ± 5 % RH, and a 12:12 h L:D regime.

(b) Experimental foods and distance treatments

We produced two synthetic foods varying only in protein-to-carbohydrate ratio (Table 1). Each food alone represented a nutritionally imbalanced diet, but when paired the two foods were complementary, because animals could obtain an optimal diet by varying their intake of the two foods. To test for effects of foraging distance on specific nutrient ingestion and resulting performance outcomes, we separated the two foods (2 cm vs. 118 cm) by placing them next to each other in small containers (18.5 cm × 13.0 cm) or at opposite ends of long containers (121.9 cm × 10.4 cm). We covered the inner sides with a thin layer of grease (1:1 mix of petroleum jelly and mineral oil), which prevented the cockroaches from walking on the sides. The short arena received one segment of egg carton for shelter (one egg-hole, Volume = ~6.062 cm^3), whereas the long arena received eight cardboard egg cartons lined length-wise (48 egg-holes, Volume = ~2900.224 cm^3). The cardboard egg cartons exactly matched the width of the containers and served to expand the three-dimensional space and hence the foraging distance. Therefore, to ingest a balanced diet, cockroaches would have to navigate over all egg cartons every time they would seek a nutritionally complementary food. Water was provided in 15.0 cm × 2.3 cm glass tubes plugged with cotton. One water tube was provided in the small arenas, and one water tube was provided in each end of the long containers plus one in the middle. Water and both foods were offered in amounts exceeding *ad libitum* intake.

(c) General procedures

All animals were weighed to the nearest mg prior to entering the experiment. They were then allocated individually to long- or short-distance containers and allowed to

feed for seven days, and remaining food plus animals were recollected. In total, 40 nymphs and 40 adult female cockroaches were tested individually in each of the two treatments, but n = 79 nymphs and 80 adults because one nymph got stuck in the grease covering the side of the container in each treatment. Foods were dried at 50 °C for at least two days before and after feeding and weighed to the nearest 10 µg. Food intake was calculated by subtracting final from initial dry mass, and specific nutrient intake was calculated by multiplying dry mass intake with the proportion of the specific nutrient in the food. The respective protein and carbohydrate intake from each food was then summed to calculate the total consumption of each nutrient. Consumption data was analyzed using MANOVA followed by *t*-tests in R 3.1.2.

(d) Nymphal growth

All nymphs (males and females) entering the experiment had molted into their 5th (last) instar within 24 hours. To obtain dry mass and lean dry mass of nymphs after 7 days in the experiment, each nymph was sexed, weighed, killed by freezing at -18 °C, dried at 50 °C for three days, and lean dry mass obtained after lipid extraction (3 days in 10 mL anhydrous ethyl ether followed by drying at 50 °C). Individual insect lipid content was calculated by subtracting lean dry mass from dry mass. Seven additional newly molted 5^{th} instar nymphs were treated in the same manner to obtain the ratio of dry mass to lean dry mass of nymphs entering the experiment. The initial individual dry and lean dry masses of experimental insects were estimated from their initial live weights using linear regressions (R^2 = 0.92 and 0.82, respectively), based on the group of nymphs that were killed at the start of the experiment. Dry mass

growth and lipid accumulation during the experiment were then calculated by subtracting the estimated initial values from the values measured at the end of the experiment.

(e) Female sexual development

All adult females entering the experiment had eclosed within 24 hours. After 7 days, females were first weighed, and then dissected and three basal oocytes were measured with an eyepiece micrometer in a dissecting microscope. The average of the three oocyte lengths was used as the individual measure for analysis. Since all basal oocytes of the German cockroach mature synchronously, there was little variation among the three oocytes within a female. Oocyte lengths were analyzed with a Mann-Whitney *U*-test because the data were not normally distributed.

RESULTS

(a) Nymphal feeding and growth

Female nymphs entering the experiment weighed 33.60±0.79 mg (mean ± SE, n = 43), and their weights did not differ between the long and short foraging distance treatments (t-test: t_{43} = -0.68, P = 0.50). Male nymphs, likewise, weighed 29.39±0.45 mg (mean ± SE, n = 36) and did not differ across the two treatments (t-test: t_{36}= 1.68, P = 0.11). Consumption of foods over the 7-day experiment was significantly greater by nymphs in the long-foraging treatment than in the short-foraging treatment for both males (MANOVA: $f_{2,33}$ = 5.0068, P = 0.012) and females

(MANOVA: $f_{2,40}$ = 10.037, P = 0.0002). Both female and male nymphs also consumed more protein in the long distance treatment than at short distance (t-test: t_{43} = 4.04, P = 0.00053 and t_{36} = 3.5956, P = 0.001039; Fig. 1a). Female nymphs also consumed more carbohydrate in the long-foraging treatment (t-test, t_{43} = 2.41, P = 0.021), but male nymphs consumed similar amounts of carbohydrate in both treatments (t-test: t_{36}= -0.19, P = 0.85; Fig. 1a). Both female and male nymphs had greater dry mass growth in the long distance treatment than at short distance (t-tests: t_{43} = 4.61, P = 0.00007 and t_{36} = 4.06, P = 0.00042, respectively; Fig. 1b). In contrast, both female and male nymphs had lower lipid accumulation relative to dry mass growth in the long distance treatment (t-tests: t_{43} = 4.61, P = 0.015 and t_{36} = -2.03, P = 0.055, respectively; Fig. 1c).

(b) Female feeding and sexual development

Newly eclosed adult females weighed 58.53±0.75 mg (mean ± SE), and they were allocated equally to the 2 treatments based on their body mass (t-test: t_{79} = -1.41, P = 0.16). While total food consumption did not vary between treatments when both diets were combined (MANOVA: $f_{2,76}$ = 0.53085, P = 0.5903), significant differences were apparent when specific nutrient consumption was analyzed separately. Adult females consumed larger amounts of carbohydrate in the long distance treatment (t-test: t_{79} = 2.12, P = 0.037; Fig. 2a), but similar amounts of protein at the two distances (t-test: t_{79} = 1.25, P = 0.22; Fig. 2a). By the end of the experiment, weight gain was higher in females in the long distance treatment than at short distance (t-test: t_{79} = 2.31, P = 0.024; Fig. 2b), and females that had foraged in the long

distance treatment grew significantly larger oocytes than females that had foraged at short distance (Mann-Whitney *U*-test: w_{79} = 1066, *P* = 0.0051; Fig. 2b).

DISCUSSION

Foragers will often modify behavior to maximize benefits (optimal nutritional energy) and minimize risk to parasitism and predation (Lima & Dill 1990). In addition, foragers must contend with food dynamics, balancing intake of specific nutrients with risks that may be associated with those nutrients, such as plant phytotoxins and other chemical threats. The decision of whether to remain on a found but inadequate food source or depart in search of more nutritious foods is one that all foragers must make. This foraging dynamic may be even more pronounced in omnivores, which are not adapted to a specific food, but rather a large host of foods that vary dramatically in abundance, toxicity, and quality.

One measure of success for a forager may be how well they regulate the intake of nutrients. All animals must consume foods at optimal ratios and in proper amounts, although this optimum may vary throughout life stage for different reasons, such as reproductive effort or sexual attractiveness. Another factor that can influence food intake is the distance between foods. It can be argued that demands associated with foraging are likely to be more significant when resources are located farther apart vs. together. Most studies on food distance have been performed on locusts, while none to our knowledge have been performed on highly mobile

omnivores, which must balance nutrient intake from foods that may be temporally and spatially transient. Generalists also tend to tolerate greater levels of nutrient imbalance compared to herbivores (Raubenheimer and Simpson 2003), making them ideal systems with which to test nutrient regulation over distance. German cockroaches are fast moving generalist omnivores that make an excellent model system to test foraging behavior due to its propensity to explore (Rivault and Cloarec 1991) and adaptive ability to compensate for nutritional imbalance (Raubenheimer & Jones 2006).

Studies on locust nymphs (Behmer et al. 2003; van der Zee et al. 2002) suggest that as the distance between complementary food dishes increases, insects switch between dishes less frequently, and fidelity to high protein food dishes increases. However, both studies did not examine long term effects of food distance on performance measures, such as growth and reproductive development. In long foraging arenas, we similarly find that cockroach nymphs consume more protein, but unlike previous studies on locusts, we also find a greater mass growth in cockroaches compared to their short-foraging counterparts. Previous studies have shown that carcass nitrogen increased with increasing protein content within foods (Raubenheimer & Jones 2006).

Behmer, et al. (2003) also found that body lipid levels of locusts increased as foraging arena increased. This unexpected result suggests that the arena sizes provided did not challenge the locusts sufficiently to cause ingested carbohydrate to

be respired. We find, however, that cockroach nymphs in long foraging arenas possessed significantly less lipid stores than those in short foraging arenas, suggesting that the distances provided were challenging enough to deplete energy reserves. Even still, distances provided for our cockroaches may pale in comparison to the distances field cockroaches regularly travel (Rivault & Cloarec 1991). It should be noted that there is likely a threshold at which foraging distances are too great, and a cockroach must navigate back to foods in known locations.

In addition to the growth benefit of long foraging arenas on nymphs, we find that adult females placed in long foraging arenas matured their oocytes faster than those in short-foraging arenas. This result was surprising as the effect of large foraging arenas on accelerated oocyte development has not been described before. While the mechanism for the beneficial effect of large foraging arenas on accelerated oocyte development is unknown, a strong correlation between juvenile hormone levels and oocyte development in adult female *B. germanica* (Schal et al. 1993) would suggest involvement of the endocrine system.

Because we only measured nutrient consumption and growth post-facto, and did not record cockroach movement, it is not possible for us to state whether longer distances forced cockroaches to limit foraging to a particular food dish, or if cockroaches expended more energy foraging in larger arenas. We considered the possibility that increased arena size would require increased carbohydrate intake for the energy required to traverse the length of the box between foods. However, our

results align with those of others that find greater protein consumption in larger foraging arenas. The question remains of why cockroaches and locusts prefer to regulate protein intake more strongly than carbohydrate. The answer in cockroach systems may reside in the unique way excess nitrogen intake is stored. Rather than excreting uric acid, cockroaches store uric acid within the fat body (Cochran 1985), which can be mobilized into usable amino acids during periods of protein shortage through symbiotic mycetocyte bacteria (Valovage & Brooks 1979). Furthermore, cockroaches may also be capable of gluconeogenesis; i.e., the deamination of non-carbohydrate sources into useable glucose. The conversion of certain amino acids into glucose has been shown to alleviate carbohydrate deficiencies in *Aedes aegypti* (Wigglesworth 1942), and the pathway of which has been found to be present in *Periplaneta americana* (Storey & Bailey 1978), though no such pathway has yet been described for *Blattella germanica*.

In this study, we find that both nymphs and adults had different patterns of food consumption in long foraging arenas, and both benefited in ways that corresponded to their life stage. Accelerated growth and sexual development due to long distance foraging has never been demonstrated before, and warrants further investigation. We suggest that German cockroaches be considered as an additional model system with which to test foraging behavior over distances.

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TABLES

Table 1

Nutritional composition of experimental diets used. Ingredients are listed with weight in grams.

Protein : carbohydrate ratio	**47 : 13**	**4 : 56**
Casein	23.5	2.05
Peptone	11.75	1.03
Albumin	11.75	1.03
Glucose	13	55.9
Cellulose	26.9	26.9
Agar	4	4
Vanderzant vitamin mixture	0.81	0.81

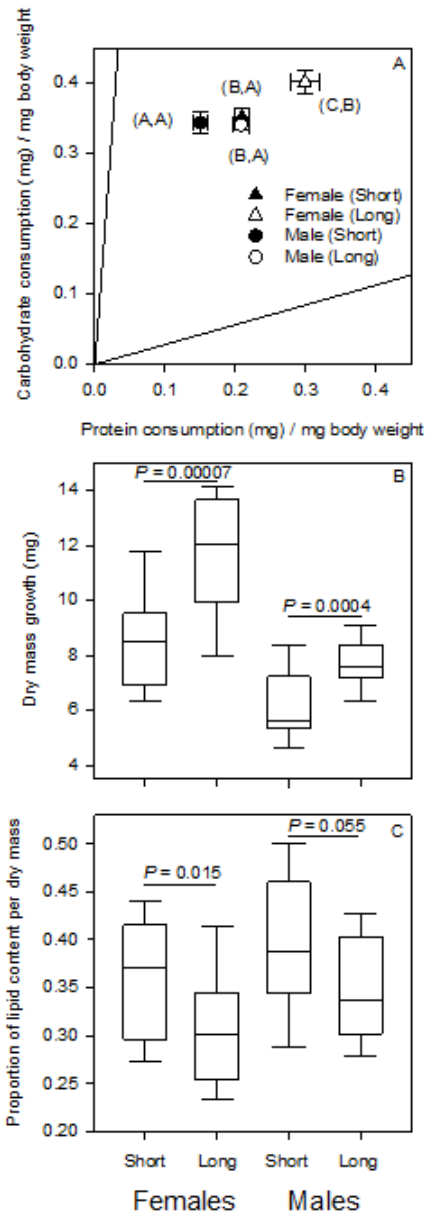

Figure 1. Effect of short and long foraging distance on female and male *Blattella germanica* nymphs after seven days of feeding during the last instar. (A) Protein- and carbohydrate-intake. Different letters indicate significant differences in protein and carbohydrate intake, respectively, within each sex (*t*-tests, $P<0.05$). (B) Dry mass growth. *P*-values are from a *t*-test within each sex. (C) Lipid accumulation relative to total dry mass growth. *P*-values are from a *t*-test within each sex. Box and whisker plots show median values with upper and lower quartiles.

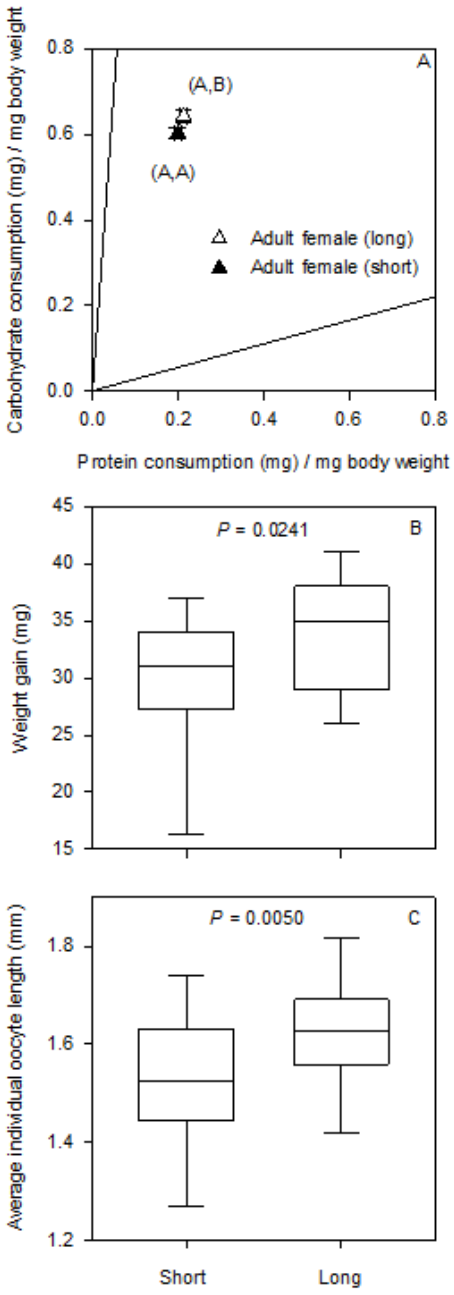

Figure 2. Effect of short and long foraging distance on newly eclosed female *Blattella germanica* after seven days of feeding. (A) Protein and carbohydrate intake. Different letters indicate significant differences in protein and carbohydrate intake, respectively (*t*-tests, $P<0.05$). (B) Weight gain. The *P*-value is from a *t*-test. (C) Average individual oocyte length. The *P*-value is from a Mann-Whitney *U*-test. Box and whisker plots show median values with upper and lower quartiles.

CHAPTER 5.

The impact of diet on male German cockroach (Dictyoptera: Blattellidae) mating success

ABSTRACT

Animals use conditional cues to determine the reproductive fitness of potential mates. German cockroaches (*Blattella germanica*) engage in a rather elaborate mating ritual prior to copulation which includes the release of sex pheromone by a sexually receptive female, male contact with female, male wing raising, and female feeding upon the male's tergal gland secretions, located on the dorsal side of the abdomen. These secretions act as an important nuptial gift to the female, responsible for arresting her long enough to engage *in copula*. During copulation, the male rotates out from under the female so that the pair is facing away from each other. During this time a spermatophore, covered in stored urates, is released by the male and enters the female's genital chamber. Oothecal production and embryo development in females have large nutritional demands, including metabolized uric acid. These male-donated urates have nutritional value and may serve as a paternal investment. Here we test the hypothesis that female *B. germanica* assess male fitness, specifically their ability to contribute urates to replenish depleted protein stores and that the diets males are fed affect their attractiveness and mating success. We first exposed virgin female *B. germanica* to carbohydrate-biased diets (Protein:Carbohydrate [P:C], 1:11), then offered them a choice of two males; one previously fed P:C 3:1, the other P:C 1:11. By using a heritable color marker we recorded male mating success. We expected that protein-starved females would primarily mate with males provided protein-biased diets. However, surprisingly, the males fed high carbohydrate P:C 1:11 diets had greater mating success than males

fed protein-biased P:C 3:1 diets. In a separate experiment, males that were first offered a choice of diets (P:C 1:11 or 1:3) had greater mating success than males restricted to high carbohydrate (P:C 1:11) diet, suggesting that males selected a macronutrient blend that improves their mating success.

Keywords:

Female choice, male attractiveness, nutrition, tergal gland, condition dependence

INTRODUCTION

Females of various species often use conditional cues to assess male quality. To be effective, these cues are often an honest indicator of a male's condition; it cannot be faked. These cues are diverse in type and number, but all largely communicate a male's condition to the female, ranging from the preferred red plumage of the male house finch (Hill 1991) and the eye width size of stalk-eyed flies (Cotton et al 2004) to the red-orange carotenoid spots in male guppies (Kodric-Brown 1989) and mud pillar building in fiddler crabs (Backwell et al 1995). In these examples, males with access to high quality diets are better able to demonstrate their quality to females in these highly visible displays.

However, other less visual cues can be used to determine a male's quality. In the field cricket, males fed high quality diets exhibited an increase in body condition and invested more in sexual signaling (Judge et al 2008), chirped at higher rates (Wagner et al 1999), exhibited more frequent calling behavior (Holzer et al. 2002) and were more attractive to females, than males fed low quality foods. In Mediterranean fruit flies, protein and sugar-fed males were more likely to copulate with virgin females (Blay & Yuval 1997), were more attractive to females in the field and achieved significantly more matings than protein-deprived males (Shelly et al 2002). Similarly, nutrient-enriched male wolf spiders enjoyed greater mating success than nutrient-deficient males, which depended upon greater courtship intensity (Lomborg & Toft 2009).

German cockroaches (*Blattella germanica*) have an elaborate courtship display prior to copulation (Rust et al. 1995). Receptive females first release a volatile sex pheromone (Liang & Schal 1993a, Liang & Schal 1993b). A male, in response, approaches and antennates the body of the female, raises its wings, and rotates 180 degrees exposing a gland located between the 7th and 8th tergites. This tergal gland contains a nuptial gift (Nojima et al 1999) shown to be high in phagostimulatory carbohydrates and lipids (Kugimiya et al. 2002, Nojima et al 1999a, 1999b, 2002), as well as amino acids (Brossut et al 1975, Kugimiya et al 2003). The female mounts the male and begins to feed on the nuptial gift within the male's tergal gland. While in the process of consuming the tergal gland secretion the attended male will engage with his left phallomere with the female's genitalia (Roth 1968, Nojima et al 1999a, 1999b, 2002, Kugimiya et al 2002, 2003, Bell et al 2007) and rotate out from under the female such that both members are facing away from each other. It is essential that females feed upon the secretion to position the female appropriately for mating. Combined, the nutrients within the tergal gland presumably allow females to assess male reproductive quality, but ultimately serve to arrest female movement long enough for males to engage their genitalia with the female (Roth 1969).

During copulation, males release a urate-covered spermatophore into the genital chamber of the female (Roth and Dateo 1964,1965). Urates are present in the fat body of both male and female *B. germanica*, and are the result of excess

nitrogenous waste (Cochran 1985). Urates act as a buffer against nitrogenous deficiencies; urate levels increase after consumption of nitrogenous foods, such as those containing amino acids, and decrease when fed low-nitrogen containing foods (Engebretson & Mullins 1983, 1986). Urates can also function as a form of paternal investment in German cockroaches (Mullins and Keil 1980, Mullins et al. 1992). During copulation, males completely empty their accessory sex glands containing uric acid onto the spermatophore (Roth and Dateo, 1964). Moreover, the significant nutrient investment by females into reproduction suggests that paternal investments of urates may play a more significant role than previously realized. Indeed, female *B. germanica* use almost all of the material ingested (~90%) prior to oviposition on a single oothecae (Kunkel 1966). This is further evidenced by the observation that food-deprived female *B. germanica* consume urate-laden spermatophores more quickly than fed females with free access to food, suggesting that these donated materials were nutritive in value to both the female and her offspring, thus serving as a form of paternal investment (Mullins and Keil, 1980). Uric acid levels of female *B. germanica* were found to significantly decrease during oogenesis and were incorporated (through the use of specialized urocyte and bacteriocyte cells) into developing embryos (Mullins and Keil, 1980).

The diets males consume have the potential to affect both tergal gland secretions and uric acid stores, both of which can affect his fitness. Because a single mating can provision a female with enough sperm for her lifetime (Cochran 1979), the potential reproductive benefit of a male that can successfully mate can be high,

although females can re-mate if the initial sperm charge is low (Cochran 1979). *B. germanica* females also assimilate significantly more urates when reared on low-protein (5%) diets vs high-protein (25%) diets, suggesting that urates obtained from males via spermatophore are assimilated as a nutrient resource by nitrogen-limited females (Mullins et al. 1992). Previous work on a separate cockroach species, *Nauphoeta cinerea*, revealed that the male diet affected lipid reserves, male sex pheromone production, attractiveness and ultimately mating success (South et al 2011). Thus, we presume that the production of a high quality tergal gland secretion facilitates mating success and that tergal gland composition is a function of male diet.

Blattella germanica deficient in one macronutrient will bias intake of that nutrient, allowing for maintenance of nutritional homeostasis (Jones & Raubenheimer 2001, Raubenheimer & Jones 2006). Because protein is required for egg development, we predict that females that are reared on foods deficient in protein will preferentially mate with males that advertise nitrogen available for sharing in urate stores. We examined two somewhat mutually-exclusive hypotheses by restricting virgin female *B. germanica* to an extremely low protein-biased defined diet (P:C 1:11) and then subsequently offering them a choice of mates; one confined to a high protein (3:1), the other a low protein (1:11) diet. We then recorded offspring paternity. If protein-limited females mated with males provided high protein diets we suspect that mate choice is determined by the female's physiological condition (macronutrient deficit) and by assessing a mate's ability to compensate for this deficit through the donation

of urates on the spermatophore. Selection by these largely protein-deprived females for mates fed a high carbohydrate diet would suggest that male mating success is enhanced by high carbohydrate diets, possibly by underlying mechanisms of higher activity and/or enhanced attractiveness of tergal gland secretions.

METHODS

Insect strains

The insects used in our study were from the Orlando Normal strain of *Blattella germanica*. This insecticide-susceptible strain, originally obtained from Orlando, Florida, has been lab maintained for over 70 years. While in our lab for the past 15 years it has been reared on rat chow (Purina 5001 Rodent Diet, PMI Nutrition International, St. Louis, MO, USA). Spontaneously generated orange body color mutants of this same strain were isolated and maintained under identical conditions. Orange body is an autosomal-recessive trait (Ross and Cochran, 1962), a color morph that is distinct from wild type black body and is useful for assessing paternity. Both rearing and experiments were performed at 25 ± 2 °C, 35 ± 5 % RH, and a 12:12 h L:D regime. All experiments were done in glass jars (10 cm diameter x 10 cm height) provisioned with glass tubes (9 mm diameter x 75 mm length) plugged with cotton to hold water.

Artificial diets

We created dry artificial diets that varied in protein and carbohydrate composition for our experiments (Table 1). Three diets were used (protein:carbohydrate 1:11, 1:3, 3:1), and all components of the diets remained the same except for the protein and carbohydrate macronutrient content. All diets were dried at 50°C for one week until at a constant weight, before use in the experiments.

Mating success of females given a choice between two males provided different diets

Females offered two males (PC 1:11 vs 3:1)

We examined the effect of diet macronutrient composition on male mating success by confining newly emerged orange-body virgin females with a diet high in carbohydrate and low in protein (P:C 1:11) content for one week. Virgin females must mate within fourteen days after emergence or a non-viable egg case will form (Rust et al. 1995). Separately, we maintained newly emerged males on one of two diets for one week: 1) black-body (wild-type)/P:C 1:11, 2) black-body/P:C 3:1, 3) Orange-body/P:C 1:11, 4) Orange-body/P:C 3:1. After pretreatment with PC 1:11 diet for one week, virgin orange-body females were given three days to mate with a male of their choice, after which time males and diets were removed and females were provisioned with rat chow and monitored daily for egg hatching. We recorded

the number of nymphs produced per ootheca and the total number of nymphs produced. Because the orange body mutation is recessive, and follows a simple Mendelian pattern of inheritance (Ross and Cochran, 1962), female mate choice was determined by examining the color of the offspring; orange colored nymphs indicated that the orange bodied female chose the orange bodied male, and black colored nymphs, or ½ black and ½ orange nymphs indicated that the female chose the black bodied male. We did not know the Black-body male genotype ahead of time, thus the mix of orange and black nymphs indicated a heterozygous male parent.

Females offered two males (PC 1:11 vs choice between PC 1:11 and 1:3)

In a separate experiment, we assessed the effect of diet choice vs the PC 1:11 diet on male mating success. To do so, newly emerged virgin females were provided with PC 1:11 diet for one week prior to contact with males. During the same week-long period, males of both color-morphs were provided with either PC 1:11 or given a choice between PC 1:11 or 1:3 diets, such that there were four male treatments: 1) black-body/PC 1:11, 2) orange-body/PC 1:11, 3) black-body/choice, 4) orange-body/choice. After the week of pretreatment, males given either of the diet treatments were presented to the female, and were allowed to mate for a three day period, after which time males were removed and rat chow was added to the jar. Females were monitored until egg hatching. Paternity of the offspring was determined using the aforementioned method of examining the color of the offspring.

Females paired with a single male that was offered one diet

To determine if differences in offspring number in the first two experiments (where females were given a choice of two males provided one of two diets: PC 1:11 vs 1:3 or PC 1:11 vs choice) were due to differential male mating success we evaluated the mating success of males given one of three diets (PC 1:11, 1:3, 3:1) using three metrics: offspring produced, time until egg hatch, and mating success rate.

Specifically, virgin adult females were given PC 1:11 diets for one week after adult emergence and before contact with males. Males, during the same one week period, were given one of three diets: P:C 1:11, 1:3, or 3:1. After the week-long diet pretreatment period, males and females were matched together to mate over the course of three days. Offspring number, time to egg hatch after male/female matching, and percent mating success was recorded.

Protein and Carbohydrate consumption by males offered paired diets (either PC 1:11 and 1:3, or PC 1:11 and 3:1)

We offered paired diets to German cockroach males to determine how they would self-select diets when given two different diet combinations. Newly emerged males were singly placed in cages with two dried pre-weighed diets (either PC 1:11 and 1:3 or PC 1:11 and 3:1) for one week, after which they were dried and reweighed. Diet

consumption was calculated by subtracting the weight of the diets remaining from the initial weight of the diets. Protein and carbohydrate consumption were calculated by multiplying the amount of diet consumed by the percentage of protein and carbohydrate present in a given diet.

Statistical analyses

We used chi-square tests to compare differences between offspring production for the experiments in which females were given a choice of two males, one fed 1:11 and the other fed either 3:1 (1^{st} experiment) or given a choice to feed between 1:11 and 1:3 (2^{nd} experiment). In the 3^{rd} experiment when females were matched with only one male, number of offspring per female and time to egg hatch was analyzed with a Kruskal-Wallis test, while percent mating success of males was analyzed using a chi-square test. Specific nutrient consumption was analyzed using *t*-tests. All averages have been expressed with standard error. All statistics were done with R version 0.98.1091.

RESULTS

Mating success of females given a choice between two males provided various diets

Of the seventy-four females tested in experiment 1, each were offered two males, fed either P:C 1:11 or 3:1. Twenty-three females (31%) produced egg cases with viable offspring. Of those, eighteen females (78.2%) mated with males provided a P:C 1:11 diet, whereas five females mated with males provided a P:C 3:1 diet (21.7%). 581 total offspring were produced by females that had chosen males given 1:11, with 151 offspring produced by females that chose males fed a P:C 3:1. This difference in offspring production was significantly different (Chi Square: X^2 = 252.5956, df = 1, $P < 0.0001$, Figure 1).

We repeated this assay, but this time females could mate with either a male given a P:C 1:11 diet or a male offered a choice between P:C 1:11 or 1:3 diet (experiment 2). Here, males given choice were free to move within the nutritional space encompassed by the P:C 1:11 and 1:3 nutrient rails. Of the eighty four (56%) females that produced offspring (out of a total of 150 females tested), 37 (44%) of these mated with males on P:C 1:11 diet, whereas 47 (55.9%) mated with males offered a choice of two diets. 1252 total offspring were produced by females that had chosen to mate with males given 1:11, whereas 1465 offspring were produced by females that had chosen to mate with males given a choice of diets. This

difference in offspring number was significantly different (Chi square: X^2 = 16.6982, df = 1, *P* = <0.0001, Figure 1).

Females paired with a single male that was offered one diet

Rather than being given choice, females were paired with males given one of three artificial diets *ad libitum*, either 1:11, 1:3, or 3:1. None of the three metrics measured significantly differed among the male diet treatments. The average time from the end of the three day mating period to egg hatch did not significantly vary among male diet treatments (Kruskal-Wallis test: X^2 = 3.7828, df = 2, *P*-value = 0.1509, Figure 2a). The average time from the end of the mating period to egg hatch for females given males fed 1:11 was 29.5 ± 1.27 days, compared to 28.94 ± 0.84 days for males fed PC 1:3, and 29.52 ± 1.05 days for males fed PC 3:1.

The average number of offspring per egg case produced by females paired singly with males fed one of the three diets also did not vary among diet treatments (Kruskal-Wallis test: X^2 = 0.2206, df = 2, *P* = 0.8956, Figure 2b). The average number of offspring per egg case produced by females mated with males fed 1:11 diets was 31.9 ± 1.099, compared to 32.37 ± 1.0 nymphs produced by males fed 1:3 diets, and 31.46 ± 1.05 nymphs produced by males given 3:1 diets.

Mating success did not significantly vary among males given any of the diets used in this experiment (Chi-square: X^2 = 2.1008, df = 2, *P* = 0.3498, Figure 2c). Of the 104

males given PC 1:11 diet, 57.7% of them resulted in successful egg hatches. 43.2% of the 125 males given PC 1:3 diet successfully fathered offspring, and 49.6% of the 139 males given PC 3:1 diet resulted in successful egg hatch when mated to females.

Protein and Carbohydrate consumption by males offered paired diets (either PC 1:11 and 1:3, or PC 1:11 and 3:1)

We tested adult male German cockroaches with two diet pairings to determine how they would self-select diets presented in the above experiments. Males given PC 1:11 and 1:3 were used in the previous experiment with female choice, and knowing how these males self-selected diets was essential to understanding why the males given diet choice were preferred over males given PC 1:11 in experiment 2.

Adult males offered a choice between the P:C 1:11 and 1:3 diets consumed an average of 0.029 mg of protein per mg of start mass (±0.00244) and an average of 0.20 mg of carbohydrate per mg of starting mass (±0.0126).

Adult males offered a choice between the P:C 1:11 and 3:1 diets consumed slightly more protein than those offered PC 1:11 and 1:3 diets. The addition of the PC 3:1 diet resulted in average consumption of 0.0378 mg of protein per mg starting mass (±0.0004) and an average of 0.2012 mg of carbohydrate per mg of starting mass (±0.0116).

Males given the two combinations of diets (1:11 and 1:3 or 1:11 and 3:1) did not significantly differ in protein consumption (T-test: t = -1.6174, df = 25.146, P = 0.1183, Figure 3) or carbohydrate consumption (T-test: t = -0.0481, df = 32.61, P = 0.962, Figure 3) per mg body mass.

DISCUSSION

We report three novel findings: 1) Male *B. germanica* fed a high carbohydrate PC 1:11 diet fathered more offspring than males fed a high protein 3:1 diet when in direct competition for a mate 2) Males given a choice of diets (PC 1:11 and 1:3) fathered more offspring than males fed solely 1:11 diets 3) Differences observed among male diet treatments in the number of offspring fathered were not due to differences in development time, offspring number per egg case, or mating success of the male given one of the three artificial diets. Together these results suggest that male nutrition can affect fitness, perhaps through the modification of his tergal gland secretion or his overall activity level. We discuss these findings in the context of how male diet may impact the delivery of key macronutrients during mating thereby affecting female receptivity and mating success. However, diet quality affects overall male fitness and our results may reflect a general competitive edge, including increased activity, enjoyed by individuals on carbohydrate-biased diets.

Much work has previously been done examining the effect of diet quality on male attractiveness (Kodric-Brown 1989, Hill 1991, Backwell et al 1995, Blay & Yuval 1997, Wagner et al 1999, Holzer et al. 2002, Shelly et al 2002, Cotton et al 2004, Judge et al 2008, Lomborg & Toft 2009). Additionally, information on the role of courtship feeding is widely available, with many studies focusing on orthopteran nuptial gift offering. Nuptial gift composition can be labile, as male bush crickets can adjust the composition of the nuptial gift to match the perceived quality of the female and maximize their paternity as a function of the perceived risk of sperm competition (Jarrige et al. 2015), and can change as a result of male nutrition, as is the case in decorated crickets (Rapkin et al 2015). These enormous nutritional investments have the potential to greatly maximize male fitness; experiments on male katydids revealed that males that donate nutrients in nuptial gifts father the offspring produced (Gwynne 1988).

German cockroaches perform a stereotyped mating ritual whereby the female releases a sex pheromone, which stimulates male wing raising to reveal the tergal gland; an organ containing phagostimulatory sugars and lipids (Kugimiya et al. 2002, Nojima et al 1999a, 1999b, 2002), as well as amino acids (Brossut et al 1975, Kugimiya et al 2003). These sugars and lipids are gustatory phagostimulants for cockroaches (Tsuji 1965, 1966). These secretions play an important role in arresting female movement long enough to allow males to assume a pre-copulatory position (Rust et al 1995, Kugimiya et al. 2002), and thus act as a nuptial gift (Nojima et al 1999a, 1999b). Failure to produce an acceptable secretion will preclude

mating, thus it is possible that German cockroach males are under considerable selection pressure to provide a satisfactory secretion for the female.

While tergal gland secretions likely do not have significant nutritive value (Kugimiya et al 2003), and only act as a nutritional arrestant, male urate stores have the potential to alleviate the nitrogen demands of reproduction in females, especially if females lack the nitrogenous resources to contribute to the developing oothecae. Females deficient in nitrogen will increase uptake and assimilation of nitrogen reserves donated by the male, and that consumption of the urates in the spermatophore is positively correlated with the level of nitrogen deficiency within the female (Mullins & Keil 1980, Mullins et al 1992). The role of protein deficiency in German cockroach mate choice is not well understood, although it has been postulated that amino acids can be detected in the tergal gland by the female (Brossut et al 1975, Kugimiya et al 2003), perhaps acting as an indicator of nitrogen reserves of the male to be gifted to the female. Here, we provide support for carbohydrate intake playing a significant role in male mating success, but we also find that some protein consumption by males in diet choice assays, improves mating performance.

In choice assays, German cockroaches prefer carbohydrate to protein-biased diets, with intake targets between P: C 1:2 and 1:3 (Jones & Raubenheimer 2001, Raubenheimer & Jones 2006, Shik et al 2014, Jensen et al 2015). Although females had been provided 1:11 diets with comparatively lower amounts of protein than they

would self-select (Jones & Raubenheimer 2001, Raubenheimer & Jones 2006, Shik et al 2014, Jensen et al 2015), male reproductive performance was improved by the 1:11 diets and, perhaps through increased quality/quantity of carbohydrate and lipid excretion in the tergal gland, dominated any protein deficiency the female may have experienced. Previous work with the cockroach *Nauphoeta cinera* has demonstrated that consuming high carbohydrate diets also produced more sex pheromone and were more attractive to females than males consuming protein-biased diets (South et al 2011). Excess carbohydrates stored as lipid reserves are the precursors of sex pheromones, and that a carbohydrate-biased diet may be preferred to improve attractiveness (South et al 2011) with attractive males mating more frequently rather than maximizing sperm production and fertility (Bunning et al 2015). Alternatively, females may not have been sufficiently protein starved as the PC 1:11 diets we provided them still, by weight, contain 5% protein. Furthermore, nutrients may be carried over between life stages, as *D. punctata* (Woodhead & Stay 1989) and *B. germanica* cockroaches (Gordon 1959, Hamilton et al 1990) can use nutrients procured in earlier developmental stages to alleviate present nutritional shortfalls. However, our diets contained the same percentage of protein used by Mullins and Keil (1980) as their low-protein treatment when they first described paternal investment of urates in *B. germanica*, so we felt that our use of these PC 1:11 diets was justified.

The results from experiment 1 (Figure 1), and previous work on male cockroach nutrition on mating success, suggested that if female preference was biased towards

males given the maximum amount of carbohydrate (PC 1:11), any introduction of diets that added an additional element of protein (such as PC 1:3) would have been expected to be deleterious to the reproductive effort of the male, as Figure 1 suggests. Because it is known that German cockroaches self-select more protein and less carbohydrate than the 1:11 food (Jones & Raubenheimer 2001, Raubenheimer & Jones 2006) (also demonstrated in Figure 3), we expected males that had been fed 1:11 to be preferred over males given any combination of diets that increased the amount of protein consumed (i.e., diet choice). Moreover, we can estimate that insects given a dietary choice between 1:11 and 1:3 or 1:11 and 3:1 foods would consume approximately 0.011 to 0.019 (respectively) mg of protein per mg body weight more than those only fed 1:11 foods (Figure 3, difference in protein consumption between self-selected ratio and the 1:11 nutrient rail). Thus, our finding that females preferred males that had been allowed to self-select their diets and consume more protein, over males forced on the previously preferred 1:11 diets, in conjunction with our results on time until egg hatch (Figure 2a), average offspring number produced per egg case (Figure 2b), and mating success of males paired alone with females (Figure 2c) did not vary among diet treatments suggests that the differences we observe may be due to differences in male attractiveness to the female, a function of his diet, and that nitrogen-deficient females may be more attracted to males that possess some nitrogenous materials to donate with his spermatophore.

However, other factors may also play a role in affecting male mating success, aside from influencing the tergal gland secretions. The consumption of carbohydrate-biased diets can have effects on lifetime fitness, besides influencing sex pheromone production. Carbohydrate consumption extends fly lifespans (Lee et al 2008, Fanson and Taylor 2012, Maklakov et al 2008), thus males consuming high carbohydrate diets may live longer, and have more lifetime opportunities to mate. Carbohydrates and lipids are energy dense, and male cockroaches consuming carbohydrate-biased diets may more aggressively pursue females. Moreover, males given diet choice may have been more physically robust compared to males fed only P:C 1:11 diet. In male wolf spiders, *Pardosa prativaga*, mating success was higher for nutrient enriched males in direct competition with deficient males, a result of their higher courtship intensity levels (Lomborg & Toft 2009). Because we did not measure courtship intensity, it is not possible for us to state at this time whether the differences we observe are due to female choice or male aggressiveness. The short distance between males given diet choice (Figure 3) and the PC 1:11 nutrient rail leads us to believe that the small difference in protein is unlikely to significantly influence general activity level, and suggests that female choice, rather than male aggressiveness, may be responsible for the differences we observe when males given diet choice are in head-to-head competition against males fed PC 1:11 for access to the female (Figure 1).

Throughout all of the mating experiments we conducted, we observed low proportion of total females tested that resulted in offspring production (43-58%), regardless of their choice of males. Although this "mating failure rate" was relatively even throughout all treatments, there may be a number of reasons why females were not more successful in producing offspring. It is possible that our PC 1:11, low protein diets may have hampered with females' ability to produce successfully hatching egg cases. Durbin & Cochran (1985) found that in the absence of an adequate food supply, German cockroach females will often reduce the percentage hatch rate of individual oothecae. PC 1:11 diets contain less protein and more carbohydrate than German cockroaches would self-select, so it is plausible that this macronutrient blend may have been perceived by females as being suboptimal to the point of sacrificing successful hatch rate. Furthermore, there may have been a possible isolation effect, as previous studies have found that German cockroach females in isolation experienced negative effects on reproduction and feeding behavior compared to those paired with other females (Holbrook et al 2000), while male maturation has been found to be relatively independent of social interaction (Uzsak & Schal 2013). Thus, had isolated females experienced delayed sexual maturity in our experiment, female sexual calling behavior, indicative of sexual receptiveness, would likely have been affected (Liang & Schal 1993b).

Our findings support the idea that the nutritional condition of females may modify behavior such that females seek out nutrients that they are deprived of to correct internal nutrient deficits. Cornelius et al (2000) reported that the physiological /

nutritional state of female oriental fruit flies affected their behavior such that they searched for food sources that relieved them of their nutritional deprivation. In particular, female oriental fruit flies in field-cage experiments that have been fed protein diets were more attracted to fruit odors than protein odors, whereas flies that had been fed fruit diets were more attracted to protein odors, rather than fruit odors. In another report, locusts had modified gustatory chemosensory responsiveness to nutrients lacking in their diets; insects fed protein-biased diets increased responsiveness to sucrose, whereas insects fed carbohydrate-biased diets increased responsiveness to amino acid mixes. Feeding behavior mirrored electrophysiological responses; diets that failed to relieve insects of their deficiencies were consumed in short duration, but diets that provided the nutrients lacking in the insects' diet were readily accepted (Simpson et al 1991). Furthermore, feeding behavior was found to be controlled by the release of neurotransmitters in *Heliothis zea*; injection of a serotonin inhibitor suppressed brain serotonin levels which resulted in greater sucrose consumption by the larvae, likewise, an increase in serotonin present in the brain was observed when larvae were fed high sucrose (carbohydrate) foods (Cohen et al 1988). Similarly, in the cockroach *Rhyparobia Madera*, insects self-selected diet ratios of P:C 1:3 even when the paired diets provided differed, demonstrating this cockroach's ability to "defend" its intake target. Injections of serotonin, however, could modify this self-selected nutrient intake ratio; injections of serotonin resulted in markedly decreased carbohydrate consumption (as the brain was tricked into thinking that carbohydrate was being overconsumed) and injections of a serotonin antagonist tricked the brain into thinking that the

cockroach was deficient in carbohydrate, causing the insects of overfeed on carbohydrate diets (Cohen 2001).

Tergal gland secretions may therefore allow protein-deficient females to gauge males for protein resources to be gained in the spermatophore. Five species of cockroach, are reported to have tergal gland secretions that contain proteinaceous material in addition to volatile components used in courtship (Brossut et al 1975). Korchi et al (1998) identified protein-secreting cells within the tergal gland of the cockroach *Leucophaea maderae*, and proteinaceous secretions that are fed upon by nymphs and adults have also been described for the cockroaches *Blatta orientalis* and *Eurycotis floridana* (Abed et al 1994). Lastly, Kugimiya et al. (2003) and Saltzmann et al. (2006) described amino acid and protein secretion in the tergal glands of German cockroach males, *Blattella germanica*, which increased through the fifth day after the post imaginal moult (Saltzmann et al. 2006), shortly before German cockroach females are expected to begin mating (Rust et al 1995). The paternal investment of urates in German cockroaches, thus may have the potential to improve the mating success of males that have nitrogenous resources to donate to females that may be lacking them, suggesting that carbohydrate intake may not be the sole determinant of a male's reproductive performance.

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Table 1. Ingredients comprising the diets used in the experiment.

Ingredients (per 100g)	1P:11C	1P:3C	3P:1C
Protein (g)	5.00	15.00	45.00
Casein (g)	2.50	7.50	22.50
Peptone (g)	1.25	3.75	11.25
Albumin (g)	1.25	3.75	11.25
Glucose (g)	55.00	45.00	15.00
Cellulose (g)	27.00	27.00	27.00
Agar (g)	9.00	9.00	9.00
Cholesterol (g)	0.55	0.55	0.55
Linoleic acid (mL)	0.55	0.55	0.55
Wesson salt mix (g)	2.50	2.50	2.50
Vanderzant modification vitamin mix (g)	0.46	0.46	0.46

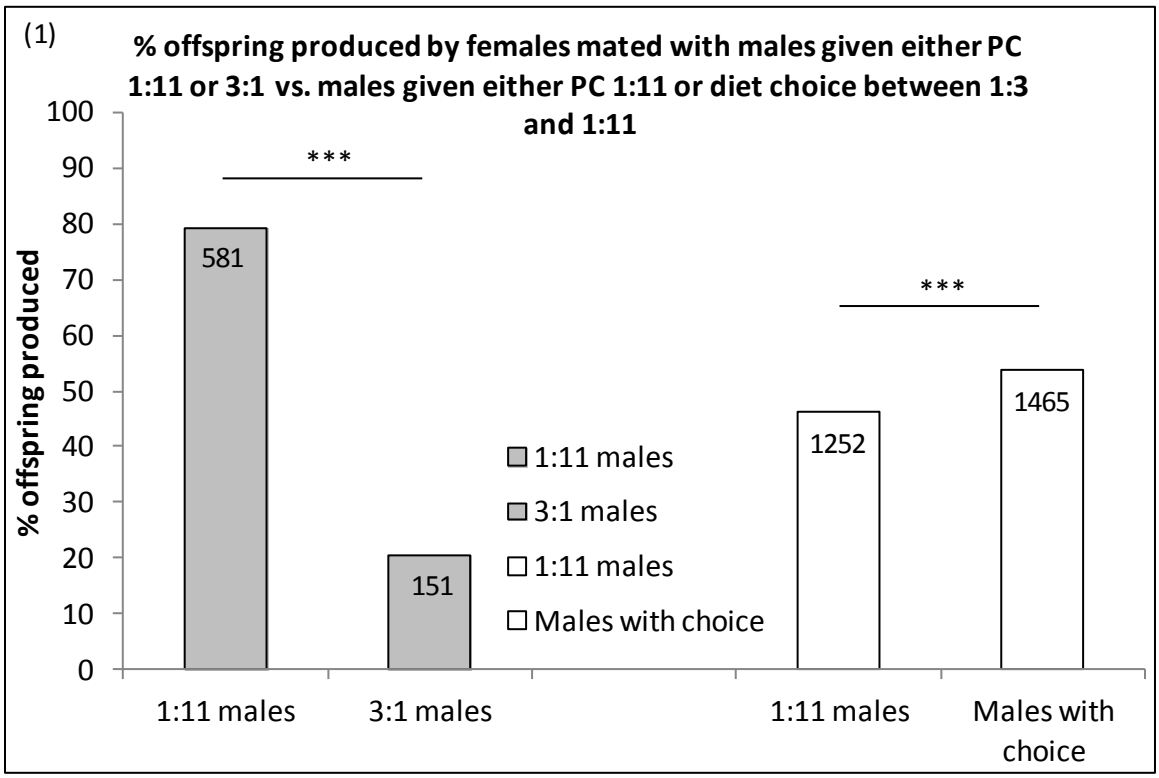

Figure 1. Percent of offspring produced by males given various diets. Grey bars (left) show the number of offspring fathered by males provided with either PC 1:11 or 3:1 diet. White bars (right) show percentage of offspring fathered by males provided with either PC 1:11 or diet choice between 1:3 and 1:11 diet. Numbers within bars indicate total number of offspring produced by males given that particular diet treatment. *P*-values determined with chi-square comparing offspring number between treatments of the same color (male pairings), with *** indicating significance below 0.0001.

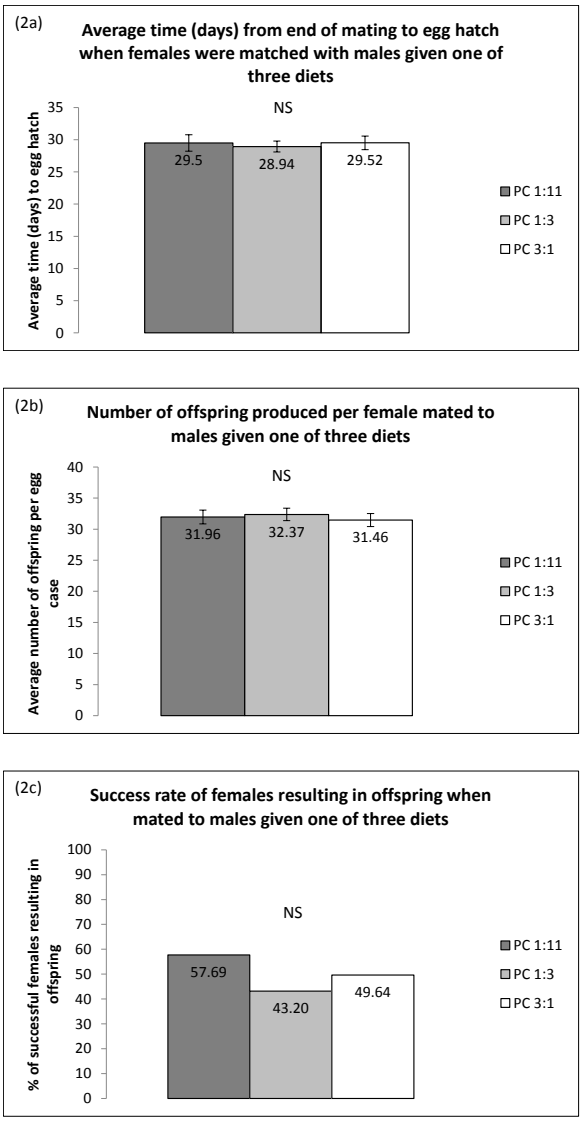

Figure 2a, b, c. Average time from end of three day mating period to egg hatch (Figure 2a), Average number of offspring per female (Figure 2b), and Success rate of females resulting in offspring (Figure 2c) when given a male fed one of three diets. Comparisons in figure 2a and b are done with Kruskal-wallis test. Comparisons of percentages in figure 2c were done with a chi-square test. NS indicates a *P*-value greater 0.05 and non-significance.

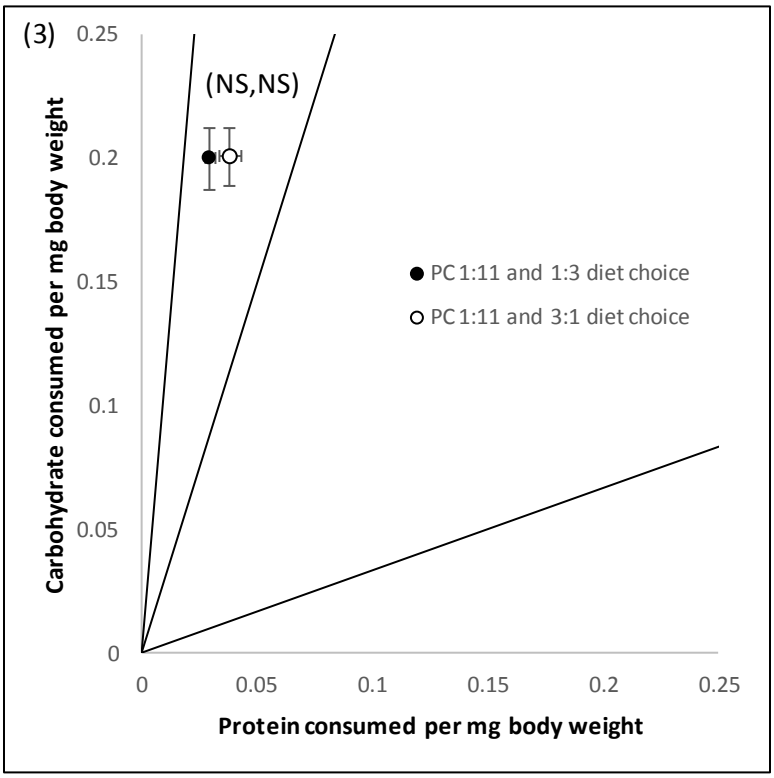

Figure 3. Consumption of specific nutrients of male German cockroaches when fed two diet combinations, either a PC 1:11 and 1:3 combination or PC 1:11 and 3:1 diet combination. Values are expressed in nutrient consumed per mg body weight. The diagonal lines represent the ratios of the three diets used, PC 1:11, 1:3, and 3:1. T-tests were conducted for both protein and carbohydrate consumption. NS in parentheses (protein,carbohydrate) indicates a *P*-value greater than 0.05 and non-significance for both protein and carbohydrate consumed.