Nitrogen fertilizer decreases survival and reproduction of locusts by increasing plant protein to carbohydrate ratio

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Abstract

Nitrogen limitation theory predicts that terrestrial plants should benefit from nitrogen inputs and that herbivores should benefit from subsequent higher plant protein contents. While this pattern has generally been supported, some herbivorous insects have shown preference and higher performance on low protein (p), high carbohydrate (c) diets as juveniles. However, little is known about the effects on reproduction in adults. Using nitrogen fertilizer, we demonstrate that high plant p:c has negative effects on Senegalese locust (Orthoptera: Oedaeleus senegalensis) reproduction and survival in an agroecological setting. For this, we measured p:c in millet plants (Pennisetum glaucum) that received two level of fertilizer (high and moderate) and a control. We found that fertilization increased p:c ratio in a concentration dependent fashion. When we caged locusts on those plants, we found that eggs laid by locusts that ate plants from the high fertilization treatment were smaller. We counted the number of locusts alive over the course of two weeks and showed that fewer females survived on fertilized plants than on control plants. Furthermore, with a laboratory choice experiment we showed that female locusts prefer unfertilized plants to plants with a high p:c. We hypothesize that this pattern will apply broadly to species that have extensive carbohydrate needs, such as long-distance migrators.

Significance statement

Nitrogen limitation theory predicts that terrestrial plants should benefit from nitrogen inputs and subsequently that herbivores should benefit from higher plant protein contents. While this pattern has generally been supported, some herbivorous insects have shown preference and higher performance on low protein (p), high carbohydrate (c) diets as juveniles. However, little is known about the effects on reproduction in adults. Using nitrogen fertilizer, we demonstrate that high plant p:c has negative effects on Senegalese locust (Orthoptera: *Oedaeleus senegalensis*) reproduction and survival in an agroecological setting (millet field, *Pennisetum glaucum*). We hypothesize that this pattern will apply broadly to species that have extensive carbohydrate needs, such as long-distance migrators.

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Introduction

The large asymmetry between plant and consumer nitrogen (N) contents (1) has led to the N-limitation hypothesis, which stipulates that in most terrestrial systems low plant N content generally impairs herbivore performance (1–3). Indeed, many studies have shown that herbivore growth, survival, and fitness respond positively to increase N or protein availability (4–11). Nevertheless a handful of studies have challenged the universality of the N-limitation hypothesis either by showing that herbivores can cope with N-deficiency, or that increasing N inputs actually decreases herbivore performance (12–16). One proposed mechanism

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to explain these exceptions is an imbalance of N (or protein) relative to carbohydrates (15). However, limited studies have tested this carbohydrate limitation hypothesis *in situ* in field settings. We propose that species with high carbohydrate demands (e.g. self-propelled migratory species) are most likely to be negatively impacted by high plant N and we tested the carbohydrate limitation hypothesis in the field using a migratory locust in a West African agroecosystem.

The importance of macronutrient balance on foraging behavior and performance has been unequivocally demonstrated in laboratory settings with artificial diets and a theoretical approach termed the Geometric Framework (17–20). For herbivores, protein and carbohydrate have been shown to be key nutrients, as illustrated by the fact that animals have evolved the capacity to regulate their intake to a specific ratio (21–23). Despite these advances, the link between macronutrient ratios selected by herbivores in the laboratory and plant nutritional content in the field has seldom been explored. Plant C:N ratios are commonly reported in the literature (1, 24–28) but not plant protein:carbohydrate ratios (for exceptions see (29, 30)). This is problematic because while N can be used as a proxy for plant protein content, the majority of plant carbon is structural (i.e. cellulose or lignin) and undigestible for most herbivores (31, 32). Furthermore, despite the demonstrated importance of both nutrients on foraging and performance, the effect of carbohydrate limitation on herbivore reproduction has yet to be tested in a field setting.

To test the carbohydrate-limitation hypothesis, we used the grass-feeder locust *Oedaleus senegalensis* in an annual cropping system (millet, *Pennisetum glaucum*) and manipulated millet protein:carbohydrate ratios using N fertilizer. We used a grass-feeder species because grasses typically rely on physical defenses like silica (33, 34) and not chemical defenses; so our treatment would affect nutrients and not secondary compounds. Furthermore, all locust species undergo long, and energetically costly, migrations fueled by lipid reserves (Hunter et al., 1981; Weis-Fogh, 1952) that come from carbohydrate consumption (Simpson et al., 2002). *O. senegalensis* is no exception and can fly up to 350 km in 24h (38–41).

To our knowledge this is the first study to measure the effect of plant macronutrient content on insect survival and reproduction in the field and a key step to linking the Geometric Framework methodology with plant nutritional landscapes in the field.

Results

1. Specific leaf area and plant protein and carbohydrate content

Five days after fertilization, there was no difference in specific leaf area (SLA) or protein content among treatments (control, moderate, and high fertilization), but carbohydrate was marginally higher in the control millet relative to the high fertilization millet (Figure 1, Table 1). After 12 days, plants from the high fertilization treatment had higher SLA than plants from the control. SLA for plants from the moderate fertilization treatment was not different from the control nor the high fertilization treatment (Table 1).

Without fertilization, plant protein content declined over time, but carbohydrate content remained constant. In both fertilizer treatments, carbohydrate content decreased through time and protein content remained constant or increased (Figure 1, Table 1). The magnitude of changes in plant nutrient contents yielded three different protein:carbohydrate ratios that were increasingly protein-biased as N input increased (Figure 1, Table 1).

1. Preference: choice experiment

Five days after applying fertilizer, locusts did not show preference when given the choice between unfertilized plants (control) and plants that received the medium level of fertilizer (medium-N). However, when given the choice between unfertilized plants (control) and plants that received high level of fertilizer (high-N), female locusts preferred control plants. Males did not show any preferences (Figure 2, Table 2).

1. Performance: survival and egg mass

More female locusts survived on the control treatment than on the high fertilization treatment (Figure 3, Table 3). Survival for males was identical on all treatments. Male wet mass, but not female wet mass, was a

good predictor of total egg mass, regardless of treatment. Females laid the same average number of eggs on all treatments; however, eggs were smaller on the high fertilization (high N) treatment (Figure 4, Table 3).

Discussion

In contrast to the N limitation hypothesis, we showed that females preferred (Fig. 2), survived longer (Fig. 3), and laid heavier eggs (Fig. 4) on plants that had lower protein:carbohydrate ratios. Males showed no difference in preference or survival to the fertilization treatments. We ran the plant preference experiment five days after fertilizing the millet. Despite there only being a marginal effect on carbohydrate content at that point (Fig. 1A), female locusts selected control plants over plants treated with high fertilizer. At that time, there was no effect of fertilization on specific leaf area (SLA). After 12 days, SLA was lower in the control treatment than the high fertilization treatment, indicating that control plants were tougher. It is thus unlikely that leaf toughness played a significant role in plant choice or locust performance in this study. After 18 days, the addition of nitrogen to soil resulted in both higher protein and lower carbohydrate levels in the millet leaves (Fig. 1), most likely because carbon was mobilized for protein building (42).

Male body size may be more important than their diet in contributing to egg size. Typically, egg size is positively correlated with the female's size and nutritional investment (43, 44); however, in this case total egg mass was correlated only with male body size (Table 3). Presumably this relationship is due to male contributing energetically to egg production *via* the spermatophore that is transferred to the females during mating. Nutrient transfer from the spermatophore to the eggs has previously been demonstrated for grasshoppers although the exact mechanism is poorly understood (45, 46). Females, relative to males, were more attuned to dietary macronutrients, potentially due to the significant impact on female survival (Figure 3) and/or the effect of female nutrition on egg size (Figure 4). Larger eggs yield offspring with higher performance traits across many species, suggesting egg size is a good correlate of fitness (44). To our knowledge this is the first time that increased plant protein:carbohydrates ratios have been shown to have a direct negative impact on reproductive output in field populations.

We hypothesize that migratory species are more likely to be carbohydrate limited because migration is energetically costly. Flying metabolic rates can be 20-100 times that of resting and insect thoracic muscles have the highest known mass-specific oxygen consumption rates (47–49). To accumulate fat reserves before and during migration animals usually rely on nutrient and diet selection (36, 49, 50). Accordingly, in the laboratory, several locust species have been reported to self-select carbohydrates-biased intakes both as nymphs and adults (18, 51) and locusts will increase carbohydrate consumption after long-distance flights (52). By contrast, a study on millet in the United States found that fertilization did not affect levels of herbivory for two non-migratory grasshopper species (53). Female locusts may have been more sensitive to carbohydrate limitation than males in this study because females likely have higher energetic demands for flight (54). Female grasshoppers, including locusts, are significantly larger than males (55). For many species migration occurs pre-reproductively (56, 57) but this is not always the case for locusts and few other insect species. For instance, female green darners (Odonata) migrate along the eastern seaboard of the United States while gravid (58). Female *O. senegalensis* (the current study species) can reproduce two to three times during their lifetime, amongst migration events, and an ootheca's mass can exceed 150 mg, 20% or more of their body mass (59). These factors may contribute to females having higher carbohydrate demands, despite the established role of protein in optimization of reproductive output (14, 60, 61).

Free-living locust populations may have difficulty securing these preferred high carbohydrate, low protein diets. Despite the study taking place in an arid environment, which are usually described as being N poor (62, 63), most plants likely do not allow locusts to meet their carbohydrate demands. We previously showed that the majority of potential host plants across all major land use types in the region (millet, groundnut, fallow, and grazing) were protein biased relative to their preferred macronutrient balance of p1:c1.6 (64). Fields with the lowest soil organic matter hosted plants with the lowest N content, which attracted the most locusts (65). *O. senegalensis* is a major millet pest; however, the millet leaves in this experiment, including unfertilized millet, were protein biased relative to locusts' carbohydrate biased demands (Figure 1; 62). For grasshoppers, including locusts, carbohydrates are more difficult to extract from plants than protein (66),

potentially exacerbating the carbohydrate limitation further.

There is increasing evidence that field populations of locusts and other animals can be carbohydrate, not protein, limited (51, 67–69). Our study illustrates a field scenario where carbohydrate limitation can negatively affect fitness, suggesting far-reaching implications for population and evolutionary dynamics. While plant carbon and nitrogen are widely recorded for ecological studies, plant carbohydrate content is rarely measured in relation to herbivore performance (70), despite the demonstrated importance of macronutrient ratio on growth, survival, lifespan, and reproduction across many taxa (14, 14, 60, 61, 71–77). This study reinforces the realization that animals have different nutrient demands based on life history and the importance of non-protein energy for herbivore communities. This research provides new insights for a complementary hypothesis to the N-limitation paradigm: the carbohydrate limitation hypothesis.

Material and Methods

A Sahel migrant: *Oedaleus senegalensis*

Locusts are grasshoppers that, in response to certain environmental cues including high population density, will shift from solitarious to gregarious phenotypes, which can subsequently lead to swarms. This phenomenon is termed locust phase polyphenism (78, 79) and is poorly understood in non-model locust species like *Oedaleus senegalensis* (80).

O. senegalensis is a grass-feeder and a major pest of millet and other cereal crops of subsistence agriculture in the Sahel zone of West Africa. Eggs start hatching with the first precipitation events of the rainy season (38), which historically occur from June through September. *O. senegalensis* typically produces three generations that all migrate following the Intertropical Convergence Zone (38). We conducted our experiments in August 2017 to coincide with the development of the first generation which is responsible for critical damages to seedling millet.

Experimental design

Our field site was the village of Nganda, in the West Central Agricultural Region of Senegal where most of millet and groundnut production takes place. The two crops are typically rotated from one year to the next. Pearl millet (*Pennisetum glaucum*) is a rain-fed crop with excellent tolerance to drought, sandy soil, low nutrient availability, and high temperatures. On August 2nd 2017 (day 0), we applied two levels of N to a millet field using urea (N:P:K 46:0:0), for a total of 3 treatments: 1) control; 2) medium N (87.5kg N. Ha-1); 3) high N (175kg N.Ha-1). For each treatment we had 4 plots of 400 m2 each.

We collected millet leaves twice during the experiment for nutrient analysis and Specific Leaf Area (SLA), first on August 7th (day 5) for both; and then on August 14th (day 12) for SLA and August 20th (day 18) for nutrient analysis. For both analyses we collected at least 30 leaves per treatment (3-5 g fresh mass) from different plants and from every plot. After collection, we kept the plants in an ice cooler until arrival at the phytosanitary base of Nganda (Direction de la Protection des Végétaux) where we photographed them over a 10 cm x 10 cm grid. We then dried them for 24-36 h in a drying oven (Kowell C1-I) at 60°C and weighed them. We recorded SLA with ImageJ (National Institute of Health). We ran the chemical assays on the dried plant material in our laboratory at Arizona State University (United States). For this, we ground plant samples for 30 seconds at 200 rpm using a Retsch MM 400 ball mill. We measured plant protein content with a Bradford assay and non-structural carbohydrate content using the phenol-sulfuric acid method, following the protocol of Clissold et al. (15). We recorded stand establishment for all treatments on August 15th (day 13) by counting the number of plants over 10 m transects, with 4 transects per plot (16 transects per treatment).

On each plot we built 3-4 cages (14-16 cages per treatment) using screening mesh (Phifer, Aluminium screen mesh). Each cage was 50 cm wide and 60 cm high, poles were 90 cm rebars, and the metallic mesh was buried underground and held by 50 cm rebars. We closed the cages with staples and binder clips. Each cage contained at least two millet plants. We calculated growth by measuring plant height in the cages or for neighboring plants on August 9th (day 7) and August 18th (day 16). We collected locusts at a nearby

location on August 7th (Minna, GPS coordinate 13º 49" 33.7N; 15º25' 00.7W). The same day, we pooled and weighed 5 females and 3 males adult *O. senegalensis* for each cage. Every 2-3 days we checked the cages and recorded mortality. On the last day of the experiment, August 21th (day 19), the remaining locusts were removed, and we scraped the first 10 cm of soil with a trowel and manually sieved the soil for eggs. Eggs were kept in alcohol and brought back to the lab to be counted, we then dried and weighed them.

We compared *O. senegalensis* preference for fertilized *vs.*unfertilized plants by conducting a choice experiment from August 7th (day 5 after fertilization) to August 10th (day 8 after fertilization). We had two treatments: control *vs.* medium N, and control *vs.* high N. For each treatment we tested 24 grasshoppers (12 females and 12 males). Locusts were hosted in a plastic cage with two leaf blades in water tubes and a perch for roosting. Plants were collected in the same fashion as for the nutrient analysis and kept in a water bucket until the beginning of the experiment. At the end of the experiment, plant material was dried, and we calculated dry consumption using a regression equation.

Statistical analysis

Statistical analyses were carried in JMP Pro 14.

Fertilization effects on plants: Specific leaf area (SLA), plant protein, and plant carbohydrates were analyzed with ANOVAs followed by a Tukey HSD when we found differences among treatments. To evaluate effects on ratio, plant protein and carbohydrate content was analyzed by MANOVA, we used a Pillai's test statistic.

Choice experiment : the dry amount of plant eaten for each treatment was compared using MANCOVA techniques; we used Pillai's test statistic. We used start mass as a covariate to correct for size differences among individuals. We included sex as an independent variable when its effect was significant. The dry amount of food eaten by males, and by females, was analyzed using ANOVAs followed by a Tukey HSD when we found differences among treatments.

Locust performance in the field: The number of locusts alive over time was analyzed by survival analysis followed by post-hoc comparison with Bonferroni correction (81). Total egg mass, egg number, and average egg mass were analyzed with ANCOVAs with pooled male and pooled female wet start masses as covariates and followed by a Student's T test when we found differences among treatment.

Acknowledgments

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Table 1 Results of ANOVA for specific leaf area, stand establishment, growth, protein, and carbohydrate content and MANOVA for protein and carbohydrate content (ratio)

Variable	Days after fertilization	Source	df	F-ratio	P-value
A) First week					
Specific Leaf Area	5	Treatment	2	1.38	0.26
Protein	5	Treatment	2	0.32	0.73
Carbohydrates	5	Treatment	2	2.95	**0.06**
Protein and carbohydrates	5	Treatment	2	0.01	0.99
B) 2nd and 3rd week					
Specific Leaf Area	12	Treatment	2	6.19	**<0.01***
Stand establishment	13	Treatment	2	2.57	0.09
Growth (D7 to D16)	16	Treatment	2	0.94	0.40
Protein	18	Treatment	2	20.39	**<0.01***
Carbohydrates	18	Treatment	2	12.88	**<0.01***
Protein and carbohydrates	18	Treatment	4	29.30	**<0.01***

Notes: Treatment refers to the different levels of fertilizer compared (control, medium N, and high N). * $P < 0.05$

Table 2 Results of MANCOVA and ANOVAs for the dry amount of plant material eaten by locusts in the plant choice experiment

Variable	Source
Dry amount of plant material eaten (g)	Treatment
	Sex
	Locust wet start mass
Comparison of dry amount of plant material eaten by each sex for each treatment	Comparison of dry amount of plant ma
Females	Ctrl vs. Medium N
	Ctrl vs. High N
Males	Ctrl vs. Medium N
	Ctrl vs. High N

Notes: Treatment refers to the two different pairing offered to the locusts (control vs. medium N, and control vs. high N). * $P < 0.05$

Table 3 Results of survival analysis for number of locust alive through time and ANCOVAs for total egg mass, egg number, and average egg mass

Variable	Source	df	ChiSq/ F-ratio	P-value
Survival				
Number of females alive	Treatment	2	14.11	**<0.01***
Number of male alive	Treatment	2	2.41	0.30
Reproduction				
Total egg mass per cag	Treatment	2	0.72	0.50

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Variable	Source	df	ChiSq/ F-ratio	P-value
	Female wet start mass	1	0.05	0.82
	Male wet start mass	1	4.39	**0.05***
Egg number per cage	Treatment	2	0.02	0.98
	Female wet start mass	1	0.22	0.64
	Male wet start mass	1	0.38	0.54
Average egg mass	Treatment	2	3.41	**0.05***
	Female wet start mass	1	1.27	0.27
	Male wet start mass	1	0.34	0.56

Notes: Treatment refers to the different levels of fertilizer compared (control, medium N, and high N). * $P < 0.05$.

Figure legends

Figure 1. Effects of fertilization treatment on millet protein and carbohydrate content after 5 days (Day 5, panel A), and after 18 days (Day 18, panel B). The dashed line marked "intake target" represents the optimal quantity of protein and carbohydrate selected by *O. senegalensis* with artificial diets (64). The other lines represent the average protein:carbohydrate ratio of millet plants for each fertilization treatment. For each treatment N=30, p<0.05.

Figure 2. Choice experiment: dry consumption (g) of unfertilized millet (control: light grey) vs. fertilized millet (medium N: grey; high N: black). For each treatment N=25, p<0.05.

Figure 3. Locust survival across treatments for 20 days. Each line represents the average number of locust alive in each cage for the three treatment. For each treatment N=112-128 locusts, p<0.05.

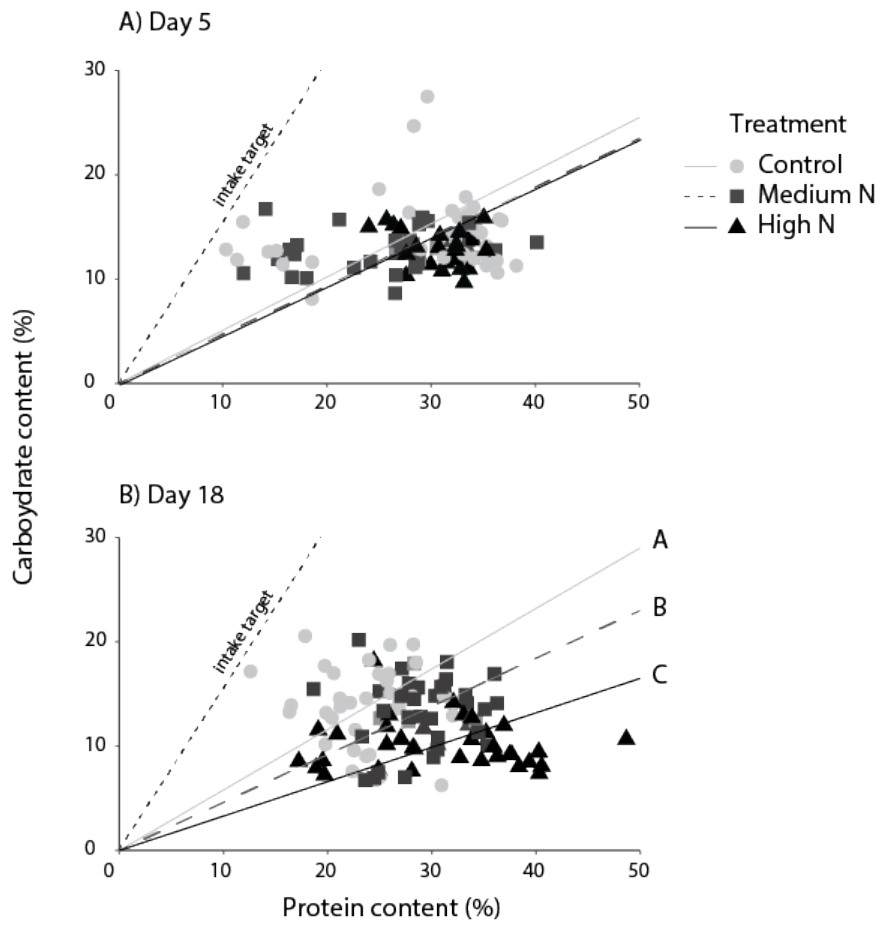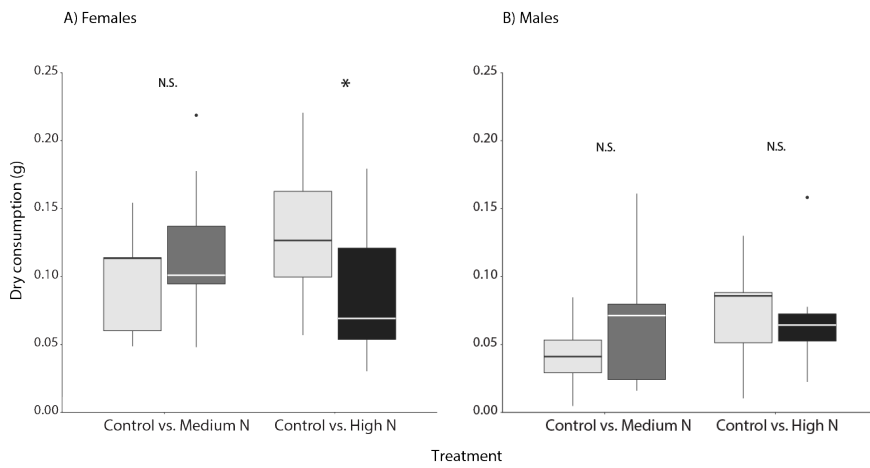Figure 4. Effect of fertilization treatment on total number of eggs per cage (panel A), and on average egg mass (panel B). For each treatment N=14 -16 cages, p<0.05.
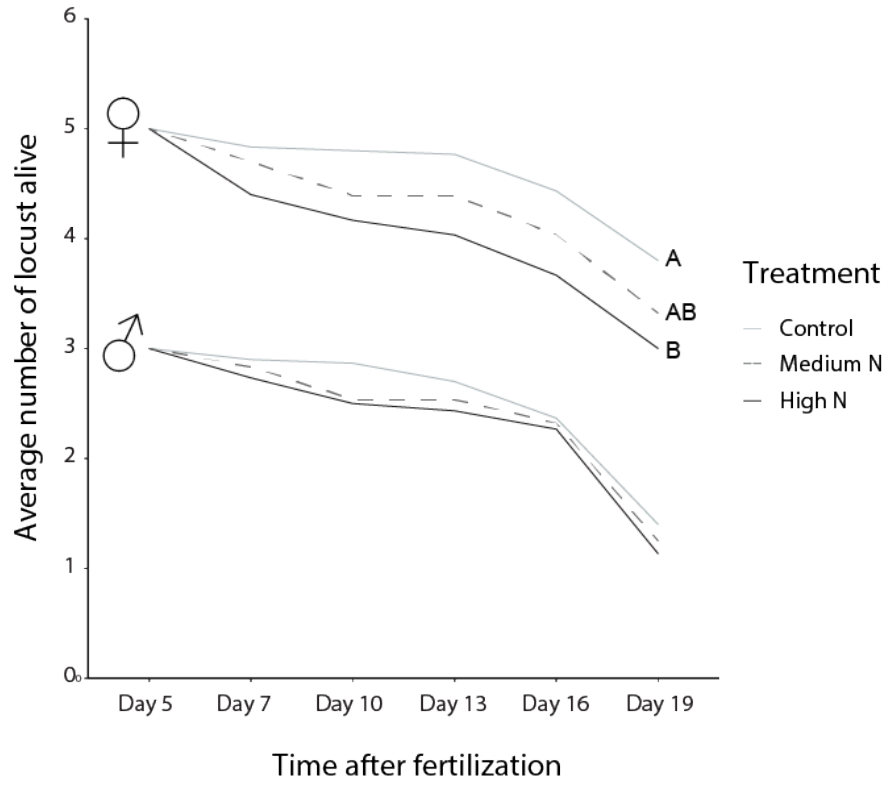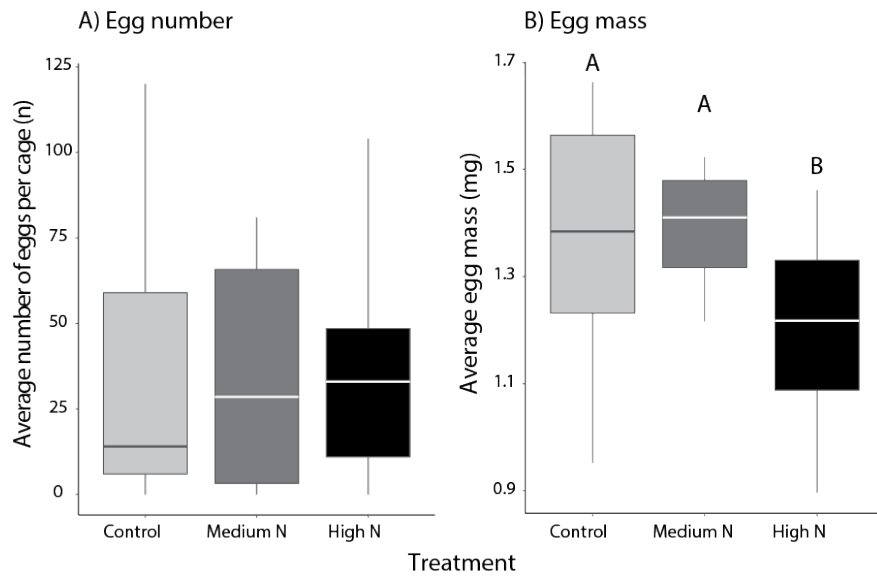
Figure 1.

Figure 2.

Figure 3.

Figure 4.