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Interaction of Neuropeptide F and diet levels effects carbonyl levels in grasshoppers

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Dietary restriction extends lifespan in a wide range of animals, especially in females (Nakagawa et al. 2012). Prolonged, life-extending dietary restriction results in a hungry animal, and this hunger likely plays a role in life-extension (Minor et al. 2009). Neuropeptide Y (NPY) in mammals, and the insect homolog neuropeptide F (NPF), are potent feeding stimulators released upon hunger (Chee and Colmers 2008) that are hypothesized to increase lifespan (Botelho and Cavadas 2015). However, the NPY/F family is pleiotropic, with some actions consistent with shortened lifespan, while others are consistent with life-extension.

Feeding is regulated in part by the NPY/F family. In mammals, NPY is immediately downstream of ghrelin (e.g., Shintani et al. 2001), increases feeding (Chee and Colmers 2008), and stimulates adipogenesis (Park et al. 2016; Shipp et al. 2016). In insects, NPF is regulated in part by the insulin-like peptide pathway (Wu et al. 2005a), and increases feeding (Van Wielendaele et al. 2013b) even on 'noxious' food (Wu et al. 2005b). Overeating, and its stimulation of the Target of Rapamycin (TOR) pathway, are well known to diminish longevity (e.g., reviews by Hoedjes et al. 2017; Kapahi et al. 2017). In this way, excessively high levels of NPY/F that are not due to hunger would be expected to produce overeating, over-activate the TOR pathway, and decrease lifespan.

Cellular maintenance also can be regulated in part by the NPY/F family (Botelho and Cavadas 2015). For example, NPY may contribute to proteostasis when it increases levels of heat shock proteins (Panossian et al. 2012). Further, autophagy in neurons is stimulated by NPY (Aveleira et al. 2015; Ferreira-Marques et al. 2016). Proteostasis (e.g., Labbadia et al. 2017) and autophagy (e.g., Woodall and Gustafsson 2018) each can positively effect healthspan in model organisms (López-Otin et al. 2013). Therefore, with regards to cellular maintenance, high levels of NPF/Y not accompanied by overeating would be predicted to increase proteostasis and autophagy, and thereby increase lifespan.

The cell maintenance benefits of NPY may be more important to lifespan than its feeding regulation effects. Chiba et al. (2014) showed that NPY-null mice do not show increased

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lifespan when on dietary restriction. NPY-null mice fed ad libitum have similar ingestion rates, insulin levels, insulin-like growth factor-1 levels, and leptin levels as wild-type controls, consistent with the notion that NPY is not necessary for stimulating cellular growth. At the same time, the NPY-null mice, even when on dietary restriction, down regulated genes involved in detoxification. This hints at an important role for NPY in damage protection to extend lifespan. Chiba et al. (2014) did not report any measures of bio-molecular damage, such as levels of oxidized proteins (i.e., carbonyls). Measures of damage are considered the best metrics of protection from oxidative stress, as they incorporate both the production of oxidizers and the activity of anti-oxidants.

Short neuropeptide F (sNPF), despite the similar name, does not appear to be closely related to the NPF family, and the roles of the two insect neuropeptides are probably not completely redundant. Most often, sNPF increases feeding rate in insects (Nässel and Wegener 2011). This functions through sirtuin2 and forkhead box (FOXO; Hong et al. 2012), which are both known to affect lifespan. However, in the desert locust (another type of grasshopper), sNPF is reported to actually inhibit feeding (Dillen et al. 2013; 2014). In the present paper, sNPF is included as a comparison to NPF.

Eastern Lubber grasshoppers (*Romalea microptera*, hereafter, grasshoppers) are an excellent model for studying the physiology of aging (Lee et al. 2015). Adult females are large enough (~6 – 8 g) to easily measure feeding rates (e.g., Heck et al. 2016), inject (e.g., Tetlak et al. 2015), and sample multiple tissues from a single individual (e.g., Judd et al. 2011). They have effective chemical defenses against bird predators (Whitman et al. 1990). Species that have strong anti-predator defenses typically are long lived (Austad, 1997), and experimentally increasing lifespan in a species that has evolved longevity is more noteworthy than increasing lifespan in a comparatively short-lived species such as mice. Dietary restriction in adult females, as 60% of the quantity of food consumed by ad libitum-fed controls, increases lifespan in grasshoppers by ~15% (Drewry et al. 2011).

For this study, the broad hypothesis is that the effects of NPY/F that are beneficial to lifespan can be separated from the effects of NPY/F that are detrimental to lifespan when overeating is prevented. To test this, injections of NPF were combined with three feeding levels, and feeding rates and carbonyls in various tissues were measured. Injections of sNPF were also tested in parallel. In particular, it is predicted that the combination of NPF injection (or sNPF injection) with dietary restriction will result in a reduction of the accumulation of carbonyls, consistent with protective effects of NPY/F, when feeding is restricted.

Methods

Animal rearing and feeding treatments

Grasshoppers were shipped as juveniles from Miami, FL, USA and reared to adulthood in the lab in Jacksonville, FL. Upon adult molt, individual females were isolated and reared in individual 500 ml containers at 35°C (light), 27°C (dark) with 14 hours of light and 10 hours of darkness and at 50% humidity (similar to Tetlak et al. 2015). All individuals were fed ad libitum until 40 days old, which allowed the first clutch to be produced and oviposited.

At 41 days old, individuals were assigned to treatment groups and experimental diets were begun (see Table 1). The feeding rate of the water & AdLib group was measured weekly. A known amount of wet lettuce was offered to each grasshopper each morning, the next morning the uneaten lettuce was collected. Then the uneaten lettuce was dried completely at 55°C and finally it was weighed. To determine the wet to dry conversion ratio, reference samples of wet lettuce were processed in the same way but not allowed to be eaten (e.g., Hatle et al. 2017). The amount eaten by the water & AdLib group was used to determine the amount of lettuce offered daily to the Full Diet and Dietary Restriction groups (Figure 1).

The amount of lettuce offered to the NPF & Full diet group, and the sNPF & Full diet group, was the same as that consumed by the water & AdLib group. The lettuce consumed by the water & AdLib group was measured regularly. This exact amount was fed to the full diet groups. Because they were injected with NPF or sNPF, the full diet groups could have been stimulated to overeat. However, because they did not have access to unlimited food, they were prevented from overeating.

During the period of peptide injections (see Injection treatments, next section), feeding rates of all ad libitum-fed grasshoppers were measured.

The rationale behind the Full Diet groups was to test whether individuals on a sufficient but not excessive feeding level, but stimulated by extra peptide, would better maintain proteins. The rationale behind the Dietary Restriction groups was to test whether individuals on an insufficient feeding level (in terms of reproductive output), and stimulated by peptide, would better maintain proteins.

Injection treatments

Daily injections were started at 73 days of age and continued for 15 days, and then grasshoppers were dissected at 88 days of age. Animals were injected late in the day, after individuals on dietary restriction completed their meals. This prevented any potential attenuation of the impact of the peptide injection by immediate feeding.

Grasshoppers were injected in the second or third abdominal segment, with 15 µl of solution delivered into the hemocoel. Water-injected animals received water, NPF-injected animals received 100 pmol of truncated NPF (YSQVARPRF-amide) in water, and sNPF-injected animals received 100 pmol of truncated short-NPF (SPSLRLRF-amide) in water (both peptides from Eton Bioscience, Inc., San Diego, CA, USA). Injections were performed with a 33-gauge syringe (Hamilton Co., Reno, NV, USA) that was alcohol and flame sterilized between animals.

Oviposition

Starting around 30 days of age, each individual was tested for oviposition twice weekly by placing it on sand (as in Tetlak et al. 2015). To calculate 'total reproductive investment', the cumulative number of eggs laid by each individual was determined, and this number was multiplied by the average wet mass of an egg (18.6 mg; e.g., Hatle et al. 2002). This total mass of laid eggs was added to the wet mass of the ovary upon dissection to calculate the total reproductive investment.

Dissections

The median lifespan for fully reproduction and ad libitum-fed grasshoppers is ~150 days (Tetlak et al. 2015). Dissections were conducted at 88 days to test animals during middle age, the period that is most important for robust self-maintenance for longevity (e.g. Levine et al. 2014). Fat bodies were removed, flash frozen in liquid nitrogen, and stored individually at -80°C . All head neural tissue anterior to the corpora allata was removed, flash frozen, and stored at -80°C ; hereafter, these preparations are referred to as 'brain'. Guts were removed and rinsed in phosphate buffered saline, then the midgut isolated by cutting just posterior to the gastric caeca and just anterior to the Malpighian tubules; each individual's midgut was rinsed, blotted dry, weighed, and then flash frozen and stored at -80°C .

Carbonyl assay

Protein was isolated from fat body and gut using the Ambion (Waltham, MA, USA) RiboPure kit, followed by a TRI reagent extraction to separate the protein and DNA. Protein was isolated from the brain using the Ambion (Waltham, MA, USA) PARIS kit. Protein was stored in 1% sodium dodecyl sulfate at -80°C .

Carbonyls were measured by immunoblot (Cell BioLabs, San Diego, CA, USA). The amount of protein in each sample was quantified by Bradford assay, and the amount of protein used for each sample was standardized. The proteins in a sample were separated by SDS-PAGE on 10% acrylamide gels. Each gel included a standard of bovine serum albumin (BSA) that was oxidized using Fenton's reagent [1 mM FeSO_4 and 1 mM HO_2 ; see example in (Heck et al. 2016)]. Proteins were wet-transferred to PVDF at -4°C using 100 V for 60 min. The membrane was treated with 2,4-dinitrophenylhydrazine (DNPH) to produce protein hydrazones from the carbonyls. These hydrazones were detected via immunoblotting with anti-DNP, which was then bound by a horseradish peroxidase-conjugated secondary antibody. SuperSignal (Thermo Scientific, Waltham, MA, USA) was used to develop the chemiluminescent blot, which was then imaged (Amersham Imager 600, GE Healthcare, Chicago, IL, USA). A representative immunoblot is shown in Figure 2. The images were analyzed by ImageJ, and the total area under the curve for all bands within a lane was summed. The relative abundance of total carbonyls in each sample was determined by comparing the area under all peaks for a lane with a sample to the area under all peaks for the lane with the BSA standard.

Protein identification

Three clusters of bands on the immunoblots were strongly carbonylated. To identify these proteins, fat body protein was separated by SDS-PAGE and the areas of interest were excised. For identification of the proteins, the gel slices were sent to the University of Florida Interdisciplinary Center for Biotechnology Proteomics and Mass Spectroscopy core.

Statistics

Feeding rate by ad libitum-fed grasshoppers during the period of peptide injection was tested by repeated-measures ANOVA. First, feeding rates were placed into 2-day periods (e.g., days 5 and 6, day 7 and 8, etc.). Most individuals had only one tested day within each

period, but when an individual was tested on both days within a period (e.g., both day 5 and day 6), the average of the two days was calculated. Repeated-measures ANOVA requires a complete data set (i.e., no missing days) for an individual to be included in the analysis. There were several missing samples in two periods (days 9 and 10, and days 11 and 12). Therefore, these two periods were dropped to obtain the best compromise between sample size and number of days included. However, feeding rates from days 9 through 12 were similar to those for days tested statistically.

Dissection data, including reproductive investment, fat body mass, hemolymph volume, and midgut mass, was analyzed with a two-way MANCOVA, using body mass at adult molt as a covariate (e.g., Moehrli and Juliano 1998). The protein carbonyl levels from all three tissues (fat body, gut, and brain) were analyzed with a single two-way MANOVA.

Results

Feeding rates

Among grasshoppers fed ad libitum, repeated injections of NPF marginally increased the feeding rate across all periods tested (repeated-measures ANOVA, Pillai's Trace $F_{3,22} = 2.58$, $P=0.079$; Figure 3). NPF significantly increased feeding in two of the four periods tested (total $n = 27$; REGWQ Multiple Range test; $P<0.05$). In particular, NPF-injection increased feeding over water-injected controls by 87% on days 5-6 and 178% on days 13-14 of injections. During the two non-significant periods, feeding by the NPF-injected group was still 38% (days 7-8) and 64% (days 15-16) greater than water-injected controls. Injections of sNPF never significantly increased feeding rate ($P > 0.05$).

Tissue sizes

All individuals were dissected on the 16th day after injections were begun, and masses of four tissues was analyzed. Diet strongly affected reproductive investment (two-way MANCOVA, $F_{2,8} = 8.84$, $P=0.0005$, Figure 4). Full diet reduced reproductive investment by 24% (REGWQ Multiple Range test, $P<0.05$) and dietary restriction reduced reproductive investment by 49% ($P<0.05$) in comparison to ad libitum-fed animals. Neither injection ($F_{2,8} = 1.82$, $P=0.173$) nor the interaction of diet and injection ($F_{3,8} = 1.23$, $P=0.307$) significantly affected reproductive investment.

Diet also strongly affected fat body mass ($F_{2,8} = 6.63$, $P=0.003$). Full diet reduced fat body mass by 28% ($P<0.05$) and dietary restriction reduced fat body mass by 52% ($P<0.05$). Neither injection ($F_{2,8} = 0.38$, $P=0.685$) nor the interaction of diet and injection ($F_{3,8} = 0.23$, $P=0.878$) affected fat body mass. These effects of diet on reproductive investment and fat body mass are similar to results of previous studies (e.g., Drewry et al. 2011; Hatle et al. 2013).

Neither hemolymph volume nor midgut mass were affected by any of the treatments (all $P>0.05$).

Carbonyl levels

Carbonyl levels across the three tissues tested were strongly affected by injection (MANOVA, Pillai's Trace $F_{6,64} = 8.42$, $P < 0.0001$), diet ($F_{6,64} = 11.21$, $P > 0.0001$), and the interaction of diet and injection ($F_{9,64} = 3.31$, $P = 0.001$; Figure 5).

Fat body carbonyls were significantly affected by the interaction of diet and injection ($F_{3,7} = 10.65$, $P < 0.0001$). Upon ad libitum feeding, both NPF injection ($P < 0.011$ Tukey's post-hoc test, adjusted for multiple comparisons) and sNPF injection ($P < 0.0001$) increased carbonyl damage over water-injected controls. In contrast, upon dietary restriction, only NPF injection decreased carbonyl damage in comparison to water-injected controls ($P = 0.002$), while sNPF injection did not ($P = 0.990$). On full diet feeding, neither NPF injection ($P = 0.209$) nor sNPF injection ($P = 0.823$) affected carbonyl levels.

Gut carbonyls were reduced by diet ($F_{2,7} = 18.04$, $P < 0.0001$). Within each treatment group, gut carbonyl levels were significantly lower upon dietary restriction than upon ad libitum feeding (all $P < 0.05$). Carbonyl levels were not reduced by full diet feeding (all $P > 0.05$). Injections also altered levels of gut carbonyls ($F_{2,7} = 7.26$, $P = 0.002$). When all diet levels were combined, NPF injection resulted in higher carbonyl levels than did sNPF injection ($P = 0.0004$). However, the only comparison within a diet level that was statistically significant was that, upon full diet, sNPF injection reduced gut carbonyls in comparison to water-injected controls ($P = 0.035$). The interaction of diet and injection did not affect gut carbonyl levels ($F_{3,7} = 0.12$, $P = 0.945$).

Brain carbonyls were not affected by diet ($F_{2,7} = 0.27$, $P = 0.763$) or injection ($F_{2,7} = 0.68$, $P = 0.511$), and were *really* not affected by the interaction of diet and injection ($F_{3,7} = 0.00$, $P = 1.00$).

Protein identification

Mass spectroscopy results suggested that the most heavily carbonylated proteins in each lane might be histones H2, H3, and H4, the 40s and 60s ribosomal proteins, histone acetyltransferase, and heat shock protein 70 (Table 2). These identifications are putative, as the isolation of the proteins by mass spectroscopy did not verify they were carbonylated.

Discussion

The hypothesis of this study, that NPY/F can have beneficial effects when separated from the detrimental effects of overeating, was generally supported. In particular, the prediction that NPF injections combined with dietary restriction would reduce carbonyl levels was supported for the fat body, but not for the gut or brain.

This study sought to determine if the life-shortening (i.e., overeating, which should activate the TOR pathway) and life-extending (proteostasis and autophagy) effects of NPY/F could be separated by controlling feeding. The present paper suggests that treatment with extra NPF without limiting feeding can increase food intake, consistent with work in other grasshoppers (e.g., Van Wielendaele et al. 2013b). Somewhat surprisingly, injection of NPF upon ad libitum feeding increased carbonyl levels in the fat body, in comparison to water-

injected controls also on ad libitum feeding. It was surprising that NPF-injection with ad libitum feeding resulted in increased carbonyl levels, as it was expected that the augmented NPF would protect from any excessive biomolecular damage upon overeating. This was not the case. It may be that activation of cellular growth pathways (e.g., TOR and insulin-like peptide) by overeating, and the damage incurred upon their activation, outcompeted any stimulation of protein protection by supplemental NPF. Work on rat livers has shown that ad libitum feeding diminished proteostasis (Gat-Yablonski et al. 2016). Taken together, overeating due to NPF supplementation resulted in increased protein oxidation. It is hypothesized that this increased protein oxidation implies an overall inhibition of the proteostasis network (*sensu* Labbadia and Morimoto 2015).

On the other hand, NPF can reduce levels of carbonyls when overeating is prohibited, consistent with life-extension. In the fat body, upon dietary restriction and augmentation of NPF levels, there was reduced carbonyls, even in comparison to water-injected controls on dietary restriction. It is hypothesized that this is due to a stimulation of the proteostasis network by NPF. This salubrious effect of NPF, revealed upon dietary restriction, may contribute to life-extension. This finding that NPF participates in maintenance of biomolecules is consistent with previous work showing that NPY-null mice had attenuated detoxification pathways (Chiba et al. 2014).

The present results are consistent with the notion that dysregulation of feeding can result in accelerated protein damage. Overeating with a sustained hunger signal (e.g., NPF & AdLib) resulted in a significant increase in carbonylation in the fat body. At the same time, the carbonyl data upon dietary restriction are consistent with the notion that hunger without eating to satiety (e.g., NPF & DR) can reduce protein damage and improve health.

Damage to biomolecules is an endpoint of cellular functions that leads to organismal aging. Antioxidant activities or gene expression levels can provide some insight on the degree and type of investment by the cell toward cell maintenance, respectively. But because production of reactive oxygen species or regulation of translation also can vary, they may not provide accurate assessments of the realization of biomolecular damage in the cell. By measuring actual levels of oxidized biomolecules, the present study assesses the outcome of all the varied components regulating protein oxidation. Because it is an endpoint, the reduction of carbonyl accumulation upon the combination of NPF and dietary restriction leads to the hypothesis that overall the proteostasis network is heightened upon this experimental treatment.

The potency of the treatment effects on carbonyl damage varied across tissues (fat body > gut > brain). This variation may be due to differences in tissues, or it may be due to how effectively the peptide (which was injected into the hemocoel at the abdomen) reached the tissue. Insect open circulatory systems are less effective at distributing biomolecules than vertebrate closed circulatory systems. Combined with the short half-life of peptides, slow circulation from the abdomen to the head may have diminished the actual signal of NPF to the brain. In contrast, the fat body is a diffuse organ in the abdomen, so it would receive a high effective dose under the protocol used here, while gut tissue would receive an intermediate effective dose. It may also be that grasshopper brain simply is less receptive to

NPF (e.g., lower density of NPF receptors). Notably, dietary restriction did not affect carbonyl levels in the brain. Near as we can tell, the brain has less plasticity in carbonyl accumulation in comparison to fat body and gut.

This study did not reveal any effects of NPF or sNPF on reproductive development, despite the fact that NPF clearly stimulates reproductive development in other grasshoppers (Van Wielendaele et al. 2013a; 2013c). Peptide injection periods of 15 consecutive days were used here, to support the major goal of measuring biomolecular damage, and this is potentially long enough to reveal effects on reproduction. However, these injections were started at a specific age (73 days, during middle age for these grasshoppers). This resulted in starting injections when the clutch cycles of female grasshoppers in the study were not in synchrony. In other words, when NPF or sNPF injections started, some individuals were just beginning a new clutch, and other individuals were about to lay a clutch. This was a result of the decision to focus rigorously on aging instead of reproduction. Because of this approach, using oocyte length or ovary mass as a metric of reproductive response to the injections in this experiment would be inappropriate. Total reproductive investment was measured, but this also includes reproduction for the 73 days prior to starting injections, so it is unsurprising that our treatments did not reveal an effect of NPF on reproductive development. The present results should not be interpreted as suggesting that NPF can not affect reproduction in lubber grasshoppers. For dietary restriction, however, our data clearly demonstrate that dietary restriction started after the initiation of reproduction reduces subsequent reproductive investment.

Upon an ad libitum diet, supplementation of NPF generally increased feeding, whereas sNPF supplementation did not alter feeding. Previous work on effects of NPF and sNPF on feeding rates has used RNAi, peptide injections, and feeding tests that use a strict five-day protocol that included a three-day fast prior to peptide injection (e.g., Dillen et al. 2013; 2014; Van Wielendaele et al. 2013b). Pilot experiments with male lubber grasshoppers showed a 38% increase in feeding upon NPF injection, in comparison to water-injected controls (Student's two-tailed t-test $P=0.065$; one-tailed $P=0.032$). In the present paper, female lubber grasshoppers were tested for increased feeding while being reared for tissue harvesting and corresponding carbonyl measurement. Thus, a three-day fast prior to testing feeding was inappropriate. That said, the feeding results in the present experiment are sufficiently robust to suggest that NPF-injection probably increases feeding, while short-NPF clearly does not affect feeding.

Across the insects, sNPF has a variety of functions. In *Drosophila*, feeding is increased by a pathway that includes sirtuin2, forkhead-box protein, and finally sNPF. A similar pathway is used for mouse NPY. This pathway homology has been used to suggest that sNPF in flies and NPY in mammals are evolutionarily conserved (Hong et al. 2012). However, other authors use sequence analyses to conclude that within the insects, NPF and sNPF are not homologous. These authors propose that the insect NPF family is homologous to mammalian NPY, and therefore has clear roles in feeding and growth promotion, but in contrast the sNPF family in insects is broadly pleiotropic, with functions that may or may not include stimulation of feeding (Nässel and Wegner 2011). Indeed, in another grasshopper species (*Schistocerca gregaria*), sNPF has been shown to *decrease* feeding

(Dillen et al. 2013; 2014), suggesting sNPF functions may be evolutionarily labile. Overall, the link between feeding stimulation, growth, and aging is less clear for sNPF than for NPF.

Despite this more tenuous link between sNPF and aging, the present sNPF results are intriguing. Carbonyl levels in response to sNPF were similar to, but less extreme than, the responses to NPF, while at the same time sNPF did not increase feeding. That said, there was only one within-diet comparison of water-injected controls and sNPF-injected animals that was statistically significant; gut carbonyls were reduced with supplemental sNPF. It may be that sNPF slightly protects gut tissue from oxidative damage, independent of feeding.

In the fat body, sNPF significantly increased carbonyl levels upon ad libitum feeding. This effect on carbonylation is similar to NPF, but sNPF did not change feeding rates. This hints at some mechanism, independent of a simple and direct response to increased energy input, that regulates oxidative damage. However, because sNPF-injection tended to *increase* carbonyl damage in the fat body in comparison to water-injection, there is only weak potential to identify a mechanism that involves sNPF that protects from damage and is independent of feeding.

Autophagy is regulated by TOR and increases healthspan. Overeating increases TOR and inhibits autophagy. At the same time, NPY/F increases feeding but also has been shown to increase autophagy in neurons (Aveleira et al. 2015). Obesity may involve dysregulation of appetite via excessive NPY/F that results in overeating. In this case, which effect wins? That is, does the overeating sufficiently increase TOR so that cells generally inhibit autophagy (which would act to shorten lifespan), or does the release of NPY/F sufficiently activate neuronal autophagy so that cells generally simulate autophagy (which would act to increase lifespan)? This question, and others like it, could be addressed by simultaneously manipulating NPY/F and feeding levels, as was done in this paper.

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Highlights

- Hunger hormones likely play a role in life-extension by dietary restriction.
- Neuropeptide F (homology of NPY in vertebrates) increases feeding and proteostasis.
- Neuropeptide F on ad libitum feeding can increase carbonyl accumulation.
- Neuropeptide F and dietary restriction reduced oxidized proteins in grasshoppers.
- Hunger hormones without eating to satiety can reduce protein damage.

Figure 1.

Diagram of injection and feeding treatments in this project. Procedures for sNPF injections are not shown but were identical to procedures for NPF injections.

Figure 2.

To measure carbonyls, protein extracts were separated by SDS-PAGE, transferred to PVDF, derivatized with DNPH to hydrazones, which were bound with an anti-DNP and developed (Cell BioLabs OxiSelect™ kit). The representative blot above shows a good run. It was selected because the treatment groups show the typical pattern of relative intensity, even though the positive control was relatively weak.

Figure 3.

Feeding rates of grasshoppers injected daily with water, truncated Neuropeptide F, or truncated short Neuropeptide F (mean $\pm$ SEs). Data are staggered to show the error bars clearly. Injections of Neuropeptide F significantly increased feeding on days 5-6, and on days 13-14 (repeated-measures ANOVA, REGWQ, $P < 0.05$). Across all four test periods, Neuropeptide F injections marginally increased feeding (Pillai's Trace, $P = 0.079$).

Figure 4.

Total reproductive investment and tissue masses and of grasshoppers after 15 days of injection treatments and 46 days of diet treatments (mean $\pm$ SEs). See text for calculation of lifetime reproductive investment.

Figure 5. Carbonyl levels in three tissues of grasshoppers after 15 days of injection treatments and 46 days of diet treatments.

Table 1.

Treatment groups of lubber grasshoppers in this study, including determination of feeding levels. N = 8 for all groups.

Treatment ID	Injection (last 15 days)	Lettuce diet (last 46 days)
water & AdLib	water	ad libitum
water & DR	water	60% of water & AdLib
NPF & AdLib	NPF	ad libitum
NPF & Full	NPF	Matched to water & AdLib
NPF & DR	NPF	60% of water & AdLib
sNPF & AdLib	sNPF	ad libitum
sNPF & Full	sNPF	Matched to water & AdLib
sNPF & DR	sNPF	60% of water & AdLib

Table 2.

Heavily carbonylated proteins from the fat body of grasshoppers. Immunoblots consistently showed three protein sizes that were the most heavily carbonylated within a lane (e.g., see Figure 2). We therefore had SDS-PAGE slices of these protein sizes analyzed for protein identification by mass spectroscopy (University of Florida Interdisciplinary Center for Biotechnology Proteomics and Mass Spectroscopy core). This analysis was not a comparison of treatment groups, but only a putative identification of heavily damaged proteins. All these proteins are commonly carbonylated.

Putative protein	Approximate size (kDa)
Histone H4	<20
Histone H3	<20
40s ribosomal protein	<20
Histone H2	<20
60s ribosomal protein	<20
Heat shock protein	<40
Histone acetyltransferase	>150
Myosin heavy chain	<150