Gene-environment interplay in *Drosophila melanogaster*: Chronic nutritional deprivation in larval life affects adult fecal output

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ABSTRACT

Life history consequences of stress in early life are varied and known to have lasting impacts on the fitness of an organism. Gene-environment interactions play a large role in how phenotypic differences are mediated by stressful conditions during development. Here we use natural allelic 'rover/sitter' variants of the *foraging* (*for*) gene and chronic early life nutrient deprivation to investigate gene-environment interactions on excretion phenotypes. Excretion assay analysis and a fully factorial nutritional regimen encompassing the larval and adult life cycle of *Drosophila melanogaster* were used to assess the effects of larval and adult nutritional stress on adult excretion phenotypes. Natural allelic variants of *for* exhibited differences in the number of fecal spots when they were nutritionally deprived as larvae and well fed as adults. *for* mediates the excretion response to chronic early-life nutritional stress in mated female, virgin female, and male rovers and sitters. Transgenic manipulations of *for* in a sitter genetic background under larval but not adult food deprivation increases the number of fecal spots. Our study shows that food deprivation early in life affects adult excretion phenotypes and these excretion differences are mediated by *for*.

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1. Introduction

The life history of an organism can often be separated into distinct stages, with discrete periods of development, that have the potential to influence later stages. The extent, direction, and permanence of these stages are dependent on a variety of internal and external factors. The environment acts as a major entity of lasting consequence on an organism's life history (Kloss et al., 2009; Marshall and Sinclair, 2010; McDade, 2012; Yang et al., 2012). Environmental factors such as temperature (Bochandanovits and de Jong, 2003; Sorensen and Loeschcke, 2001), social environment (Bagot et al., 2012; Krupp et al., 2008; Levine et al., 2002; Schneider et al., 2012), and nutritional composition (Burns et al., 2012; Fitzpatrick et al., 2007; Scrimshaw, 1998) influence early life experience and affect adult fitness.

Nutritional stress can occur on acute or chronic time scales. Studies investigating acute nutritive stress focus on starvation resistance during short, discrete developmental time periods (Lin et al., 2013). Studies that investigate chronic nutritional stress

focus on long-term consequences of nutritional stress within (Kaun et al., 2007a) and between life history stages (Burns et al., 2012), throughout life (Kristensen et al., 2011), as well as in natural selection experiments performed on evolutionary time scales (Kloss et al., 2009; Sokolowski et al., 1997). Nutritional composition of an environment has the potential to vary in uniformity, quality, and complexity. Ratios of nutrients and total quantity of calories available within and between habitats can be experimentally manipulated (Piper et al., 2005; Skorupa et al., 2008). The ability to thrive in a chronically nutrient restricted environment can be evolutionarily advantageous (Vijendravarma et al., 2012a). This suggests that gene-environment interactions contribute to how well negative stressors are buffered. In addition, gene-environment interactions early in life may influence response to adversity later in life (Boyce et al., 2012).

In the current study we use *Drosophila melanogaster*, a genetically tractable model organism, to investigate the effects of nutritional stress. *D. melanogaster* has a complex life cycle with distinct larval and adult stages. Here we investigate the effects of chronic food deprivation in the larval and/or adult periods of development on adult excretion by using the rover and sitter natural allelic variants of the *foraging* (*for*) gene (de Belle et al., 1989; Osborne et al., 1997). The *for* gene was originally discovered through mapping phenotypic variation in larval movement

patterns on a yeast water paste (Sokolowski, 1980); rover larvae move more while foraging for food than sitter larvae (Sokolowski, 2001). *for* encodes a cGMP-dependent protein kinase G (PKG) which underlies the extensive pleiotropy and plasticity in both larval and adult *D. melanogaster* behavioral and metabolic phenotypes (Burns et al., 2012; Kaun et al., 2007a; review Reaume and Sokolowski, 2009). When adult heads and larval nervous systems are assayed, rovers have higher *for* mRNA expression levels and PKG enzyme activity levels than sitters (Kaun et al., 2007a; Osborne et al., 1997). A previous study showed that chronic larval but not adult food deprivation interacts with the *for* gene to affect darting-exploration defined as the extent to which adult flies move from the edge of an open field and dart into the center (Burns et al., 2012). The above studies suggest that *for* interacts with the nutritional context to affect a number of phenotypes.

In the current study, we investigate the adult excreta of rovers and sitters under varying nutritional regimes in the larval and adult life history stages using a protocol adapted from Cognigni et al. (2011). Quantifying excretion phenotypes allows for a visual readout of visceral processes that are associated with nutritional need, regulation, and function (Cognigni et al., 2011). Excretion analysis can act as a proxy for gut function providing information about a highly nutrient sensitive tissue (O'Brien et al., 2011) and a hub for whole-animal nutrient homeostasis (Cognigni et al., 2011). PKG has been associated with many phenotypes arising from function both inside (Burns et al., 2012; Donlea et al., 2012; Kaun et al., 2007b; Kuntz et al., 2012; Mery et al., 2007; Wang et al., 2008) and outside the brain (Dow et al., 1994a,b; Klein et al., 2013). However, little is known about the interplay between allelic variation in *for* and early life nutritional adversity on adult excretion phenotypes.

2. Materials and methods

2.1. Fly strains

The homozygous *for^R* (rover) and *for^s* (sitter) strains described in Fitzpatrick et al. (2007) were reisogenized in 2013 and their first and third chromosomes were made co-isogenic.

Transgenic expression of *for* was accomplished using the UAS-GAL4 system (Brand and Perrimon, 1993). A *UAS-forT1* line described in Donlea et al. (2012) and Kuntz et al. (2012) were crossed to a *hs-GAL4* line described in Kaun et al., (2007a). The UAS line was backcrossed into a w^{118}; *for^s* genetic background using 9–10 generations of backcrossing after which the *X*-chromosomes were replaced with wild-type *for^s* *X*-chromosomes to remove any phenotypic effects of w^{118} alleles. The *hs-GAL4* line was also on a *for^s* genetic background. We crossed the UAS line to the *hs-GAL4* line and raised the test populations in the nutrient treatment conditions (described below) for the excretion assay at 25 °C as in Kaun et al. (2007a). Controls were crosses of each of the GAL4 and UAS lines with the isogenized *for^s* strain.

Strains were maintained in 6 oz bottles on a 45 mL standard yeast-sugar-agar medium (1000 mL H_2O, 100 g sucrose, 50 g Fleischmann's yeast, 16 g agar, 8 g $C_4H_4KNaO_6$, 1 g KH_2PO_4, 0.5 g NaCl, 0.5 g $MgCl_2$, 0.5 g $CaCl_2$, 0.5 g $Fe_2(SO_4)_3$) at 25 °C with 40–60% humidity under a 12 h L:D cycle with lights on at 0800 h (standard conditions).

2.2. Nutritional treatments

Standard food medium was designated as high (H) and nutrient deprivation food medium was designated as low (L). Low food consisted of an 85% reduction in both yeast and sugar compared to standard food. Larval and adult flies were exposed to the following combinations of L and H food: HH, LH, HL LL, with the first letter showing the quality of food available throughout larval develop-

ment and the second letter showing the quality of food available during the adult stage.

2.3. Excretion assay

The protocol for measuring excretion in adult flies was modified from Cognigni et al. (2011). To prepare adult flies for the excretion assay, 20 first instar larvae ±2 h in age were seeded into 50 mL vials containing 10 mL of L or H food medium. Larvae developed in incubators under standard conditions. Flies eclosed between 9 and 14 days post-hatch depending on the nutrient treatment with the LL and LH treatment flies taking longer to eclose. Adult flies were transferred ±5 h post-eclosion (O'Brien et al., 2011) via light CO_2 anesthetic into L or H food medium vials depending on the nutrient treatment they were randomly assigned to. All adult food treatments included 0.5% Bromophenol blue (BPB). Male and female adult flies were housed separately with *n* = 20 flies per vial. Mated female test flies were generated by housing 14 females with 6 males for 3 days and it was unknown at which time point mated status was achieved.

When flies reached 3-days-old, four adult flies (virgin female, mated female or male) were aspirated into each 60mm × 15 mm petri dish. Each dish contained a food puck (1 cm³, supplemented with 0.5% BPB) made from L or H food according to adult treatment. The sample size for the excretion assays consisted of 30 dishes per sex/condition (virgin female, mated female or male) and per food treatment (HH, LH HL, LL).

For the excretion assay, petri dishes were labeled in a double blind procedure and placed lid side down alternating between empty and treatment dishes in a randomized position in wire cages complete with an empty petri dish border. Dishes remained undisturbed in an incubator at standard conditions for 24 h ± 0.5h. Flies were then discarded and petri dish lids and bottoms were scanned using an HP LaserJet scanner at a high-resolution (1200 × 1200) color setting. Images were saved as Jpeg files and adjusted for color, brightness, contrast, and exposure with Adobe Photoshop. Images were analyzed by counting the total number of fecal spots and visually analyzing differences in the shape of excretion per petri dish (lid and bottom). Differences in excrement shape were visually quantified as large and round (round) or small, thin, and rod shaped (Reproductive Oblong Deposit – ROD) as described in Cognigni et al. (2011).

2.4. CAFÉ assay

We modified the CAFÉ assay from Ja et al. (2007) to quantify the volume (μL) consumed over a 24 h period by mated female rovers, sitters, transgenic flies and controls who experienced all combinations of nutritional environments (HH, LH, HL, LL). Larvae and adults were reared as described above. Three-day-old mated adult females were individually aspirated into the CAFÉ assay and allowed to acclimatize for 24 h prior to quantification of their food intake in the 24 h CAFÉ assay. The CAFÉ assay took place in an environmental chamber at standard conditions with *n* = 30 per treatment. Flies that did not feed in the CAFÉ assay were not included in the analyses. The proportion of flies that did not feed during the CAFÉ assay did not differ between strains and treatments. Flies were housed individually in 50 mL vials with 20 mL water and packed cotton to prevent desiccation. A calibrated glass micropipette (5 μL) filled with liquid food medium by capillary action was inserted into the lid of the test vial. Two different CAFÉ assay liquid food treatments were used to complement the H and L excretion assay conditions. High CAFÉ assay food (CAFÉ H: 100 mL H_2O, 10 g sucrose, 5 g autolyzed yeast) and low CAFÉ assay food (CAFÉ L: 100 mL H_2O, 1.5 g sucrose, 0.75 g autolyzed yeast) were modified to contain 0.5% BPB dye and matched to adult nutrient conditions. Multiple control capillaries were used in each test row to determine evaporative losses of both CAFÉ H and

CAFÉ L foods, which were averaged per row and subtracted from experimental readings.

2.5. Statistical analysis

Data was analyzed using ANOVA. Mean ± s.e.m. are shown in all figures. $P < 0.05$ was considered significant. All ROD:Round ratio data was $\ln(x + 1)$ transformed prior to analysis.

3. Results

3.1. Number of fecal spots excreted

We first investigated whether the nutritional environment in larval and/or adult life affected the mean number of fecal spots excreted by adult mated males, mated females and virgin female flies. Animals were given either low (L) or high (H) quality food during the larval and adult periods of development in four treatments (HH, LH, HL, LL). As described above, the larval period was designated by the first letter and the adult period designated by the second letter. The number of fecal spots was measured for both rovers and sitters.

We found that both nutrient treatment and strain influenced the mean number of fecal spots excreted by adult flies. The adult nutrient treatment had a highly significant main effect on mean number of fecal spots excreted, as did strain (Fig. 1a and b). Significant interactions included strain-by-larval nutrition, sex-by-adult nutrition, and larval nutrition-by-adult nutrition (Fig. 1a and b).

3.2. Strain difference

We wondered if a specific nutrient treatment was responsible for the significant strain effect found. Our quantitative food analysis of *D. melanogaster* adult excretion revealed that when nutrient deprived as larvae and well fed as adults (LH), both mated female and male rovers showed a significantly higher mean number of excreta than their sitter counterparts (Fig. 1a and b). This was also found for virgin females (mean ± s.e.m: sitter = 7.712 ± 0.942 and rover = 12.916 ± 1.997, $F_{(1,46)} = 5.544$, $P = 0.023$). Thus, strain differences in excretion between rovers and sitters only occurred in the LH treatment.

3.3. CAFÉ assay

Cognigni et al. (2011) showed that when the adult food environment was manipulated, the number of fecal spots excreted was a
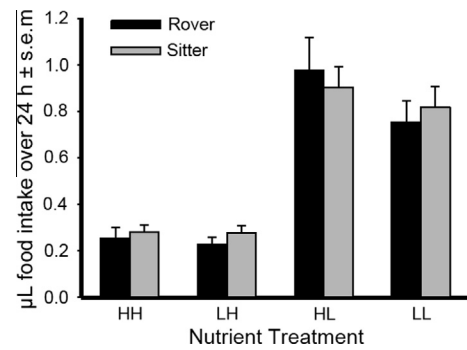

Fig. 2. CAFÉ assay: mated female flies fed low quality food as adults consume significantly more food in a 24 h period regardless of strain. Histograms show mean ± s.e.m food intake (μL) in a 24-h period. ANOVA showed significant effect of food intake in mated females between different adult nutrient treatments ($F_{(1,217)} = 136.025$, $P = <0.0001$). No significant strain differences were detected ($P > 0.05$).

function of the quantity of food ingested, and that nutritional quality was inversely related to both the amount consumed and excreted by adult flies. We explored this relationship in rover and sitter flies in the context of early life nutritional adversity by investigating the effect of larval and adult food quality on adult food consumption. We found a significant effect of adult nutrition quality on food intake in adult flies regardless of strain (Fig. 2). This pattern was also found when the transgenic flies tested in Section 3.4 (below) were tested in the CAFÉ assay ($F_{(1,334)} = 113.500$, $P = <0.0001$). All flies provided with low adult nutrition showed higher food intake in the CAFÉ assay. In summary, we found no significant effect ($P > 0.05$) of strain (rover vs. sitter; transgenic vs. control) on the mean quantity (μL) of food consumed over the 24 h test period in any treatment condition.

3.4. Transgenic rescue

To investigate whether *for* plays a direct role in the observed differences in excretion we used the UAS-GAL4 system (Brand and Perrimon, 1993) to manipulate *for* expression. As described above, we expressed *UAS-forT1* using a *hs-GAL4* at 25 °C. We were particularly interested in the results of experiments in the LH nutrient environment, the condition where rover/sitter differences were found. We found that the ubiquitous expression of *for* significantly increased the mean number of fecal spots relative to controls (Fig. 3). Transgenic manipulation of *for* expression within
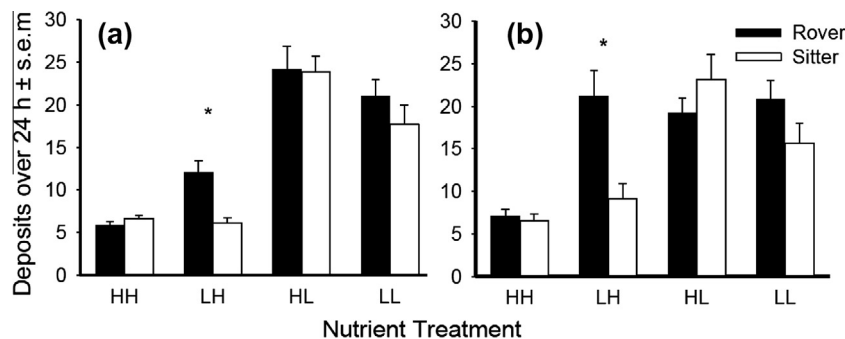

Fig. 1. Rover and sitter strain differences in deposits were found for adult flies that were chronically food deprived throughout their larval period but well fed as adults. The *x*-axis shows nutrient treatments that were either high quality food (H) or low quality food (L) where L = 85% reduction sugar and yeast compared to the normal diet. The *x*-axis labels denote the larval (first) and adult (second) nutrient conditions respectively. All histograms show mean ± s.e.m deposits in a 24-h period. ANOVA showed a significant effect of strain ($F_{(1,460)} = 8.35$, $P = 0.004$), adult nutrition ($F_{(1,460)} = 133.960$, $P = <0.0001$), strain-by-larval nutrition ($F_{(1,460)} = 14.79$, $P = <0.0001$), sex-by-adult nutrition ($F_{(1,460)} = 7.26$, $P = 0.007$) and larval nutrition-by-adult nutrition ($F_{(1,460)} = 22.97$, $P = <0.001$). Overall, deposits were higher when adults received the L compared to the H food treatment. Between strain within food treatment conditions, shown with an asterisk, were found: (a) in mated females significant differences between strains occurred in LH when larval nutrition was poor and adult nutrition was high ($F_{(1,50)} = 12.173$, $P = 0.001$) and (b) in males significant differences between strains also occurred in LH when larval nutrition was poor and adult nutrition was high ($F_{(1,54)} = 10.603$, $P = 0.002$). * Denotes $P < 0.01$ between strains within LH treatments.
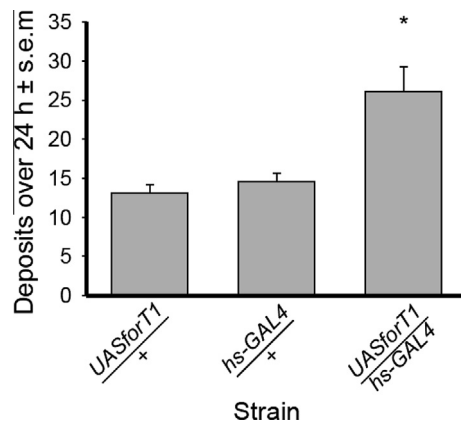
Fig. 3. Low-level ubiquitous *for* expression on a sitter genetic background resulted in an increase in excretion in a LH nutrient environment. Nutrient treatment was LH, which was low quality food (L) during larval development and high quality food (*H*) during adult life stage. L = 85% reduction in nutrients. This was the condition where rover/sitter differences were found. All flies tested were mated females. All histograms show mean ± s.e.m deposits in a 24-h period. ANOVA showed a significant effect of strain between transgenic rescue and controls ($F_{(2,63)}$ = 16.894, $P < 0.0001$). * Denotes $P < 0.01$ between strains within LH treatments. Controls were tested as heterozygotes. cDNA was *UAS-forT1*, GAL4 was *hsGAL4*. Transgenic manipulations were done on sitter genetic backgrounds (+).

other nutrient treatments (HH, HL, LL) did not significantly affect the number of fecal deposits between strains ($P > 0.05$).

3.5. Shape difference

A previously reported difference in the shape of excreta (Apger-McGlaughon and Wolfner, 2013; Cognigni et al., 2011) was confirmed in our experiments (Fig. 4a and b). Round deposits were previously characterized as more watery and less concentrated relative to RODs, which were more concentrated and differently shaped. We also confirmed that ROD production was known to be coupled with physiological changes that occur in females post-mating, such as water retention, explaining this more concentrated appearance (Cognigni et al., 2011). Another qualitative observation noted differences in pH of round excreta, as was previously described in Cognigni et al. (2011).

To investigate this observed shape difference further, we quantified the ratio of ROD and Round deposits for each of our nutritional treatments in mated females and males. Our results demonstrated that ROD:Round ratio was largest in mated females that were well fed throughout their larval and adult lives (HH). No significant effect of strain on ROD:Round ratio was found (Fig. 4c). As expected, ANOVA showed a significant effect of sex. Both larval and adult nutrition significantly affected excreta shape, with the presence of a low quality food environment at any stage of development being associated with a lower ROD:Round ratio. Significant interactions included strain-by-adult nutrition, sex-by-larval nutrition, sex-by-adult nutrition, larval nutrition-by-adult nutrition, and sex-by-larval-by-adult nutrition (Fig. 4c).

4. Discussion

4.1. Larval nutrition, adult excretion, and *for*

Low adult nutrition increased excretion of both rovers and sitters to a similar extent. In contrast, the LH treatment uncovered rover/sitter differences in adult excretion. When low larval nutrition was combined with high adult nutrition an increase in the adult excretion of rovers but not sitters was found. This suggests that from the perspective of adult excretion, the allelic expression
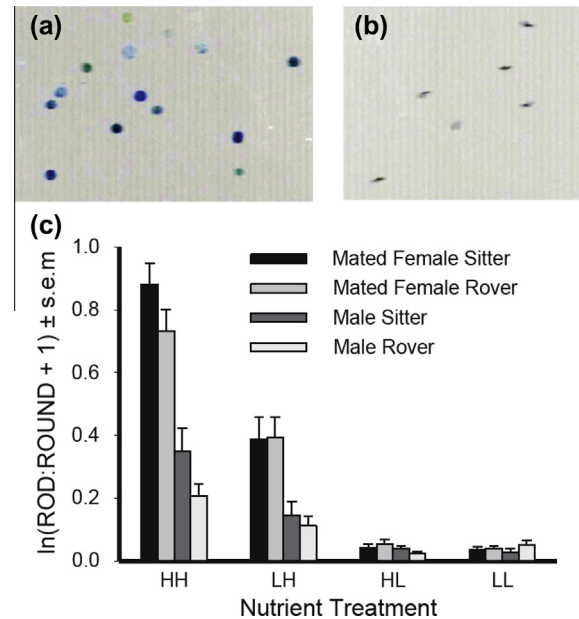

Fig. 4. Qualitative analysis of mated female excreta revealed ROD and round shaped excreta. No strain differences were found in excreta shape. Shape differences in excretion of mated females: (a) round deposits are more watery and less concentrated, (b) Reproductive Oblong Deposits (RODs) are more concentrated and production is coupled with physiological changes that occur in females post-mating (Cognigni et al., 2011). (c) ROD:Round ratio was largest in mated females that were well fed throughout their larval and adult lives (HH). No significant differences were found between strains in ROD:Round ratio. All histograms show mean ± s.e.m. ANOVA showed a significant effect of sex ($F_{(1,460)}$ = 86.04, $P < 0.0001$), larval nutrition ($F_{(1,460)}$ = 42.87, $P = <0.0001$), adult nutrition ($F_{(1,460)}$ = 277.948, $P = <0.0001$), strain-by-adult nutrition ($F_{(1,460)}$ = 3.936, $P = 0.048$) sex-by-larval nutrition ($F_{(1,460)}$ = 10.63, $P = 0.001$), sex-by-adult nutrition ($F_{(1,460)}$ = 22.97, $P = <0.0001$) larval nutrition-by-adult nutrition ($F_{(1,460)}$ = 41.687, $P < 0.0001$), and sex-by-larval-by-adult nutrition ($F_{(1,460)}$ = 8.24, $P = 0.004$).

of *for* found in sitters may somehow have buffered adult flies from larval nutritional deprivation. Support for this hypothesis comes from our finding that when *for* was transgenically increased in sitters in the LH condition, excreta significantly increased. Further investigations are needed to determine how excretion differences in rover and sitter flies are regulated. This will require an understanding of the tissue and developmental specific roles *for* plays in excretion under different larval nutrient conditions. The broad expression pattern of *for* including the nervous system, trachea, and gut provides us with candidate tissues to further these investigations (Chintapalli et al., 2007; Graveley et al., 2011).

How *for* buffers early life nutritional stress with regards to excretion behavior is not known. Kaun et al. (2007a) showed that rover and sitter time from egg-hatch to eclosion differed under the chronic larval food deprivation conditions used in the current experiment. Sitters took longer to eclose when the nutritional value of food was limited and ubiquitous expression of *for* in a sitter genetic background restored time to eclosion to a rover level. The lengthened developmental time of sitters under larval nutrient stress might play a role in buffering the effects of early life stress on adult excretion phenotypes.

Our results suggest that natural selection under conditions of chronic larval food deprivation may result in differences in adult excretion. Sitters may be better buffered to low larval nutrition than rovers. Kloss et al. (2009) performed a long-term natural selection experiment under conditions of chronic larval food deprivation and in a follow up study Vijendravarma et al. (2012b) found that sitter larval foraging phenotypes were selected in these experiments. This change in phenotype may have arisen from chronic larval food deprivation affecting adult excretion phenotypes.

Future experiments will determine whether the gene-environment interactions reported here have fitness consequences.

4.2. Early life nutritional stress does not affect adult ingestion in the CAFÉ assay

Our analyses of adult fly ingestion in the CAFÉ assay revealed no significant differences in rover and sitter flies reared under our nutritional treatment conditions. This suggests that the different excretion responses of rovers and sitters to the LH treatment cannot be explained by differences in food intake as measured in the CAFÉ assay in the current study. Another hypothesis is that internal differences, arising from the LH treatment, may contribute to rover/sitter excretion differences found in adults reared under LH conditions. These internal differences could, for example, arise from structural or functional differences in gastrointestinal regulation. Kaun et al. (2007a) showed that under chronic larval food deprivation rover larvae exhibited higher glucose absorption in their guts than sitters and that *for* mediated these differences in larval gut absorption.

Interestingly, recent research has shown that *D. melanogaster* gut growth and regulation are not solely governed by the gut. Inter-organ relationships exist, for example between the gut and trachea (Li et al., 2013) and the gut and fat body (Colombani et al., 2003). These inter-organ relationships, such as the gut–trachea, have been shown to be developmentally plastic depending on variation in the composition of the nutritional environment throughout life (Linneweber et al., 2014). In addition, in insects *for*'s gene product, cGMP dependent PKG is present in the gut (Graveley et al., 2011; Tobback et al., 2013) as well as the interfacing organs with which cellular signaling occurs such as the malpigian tubules (Dow et al., 1994a, 1994b), trachea, (Chintapalli et al., 2007; Graveley et al., 2011) and fat body (reviewed in Haunerland and Shirk, 1995). Future studies will investigate the involvement of *for* in gut development and regulation under larval food deprivation conditions.

4.3. ROD:Round ratio and reproduction

ROD production is associated with post-mating physiological changes in females mediated by male sex peptide (Liu and Kubli, 2003; Cognigni et al., 2011) and egg production (Drummond-Barbosa and Spradling, 2001). Both the action of male sex peptide and the impairment of egg production in low nutrient environments provide likely explanations for the sex specific nature of the observed shape differences in excreta, while our results suggest that *for* expression is not highly influential on excreta shape. Our finding that ROD:Round ratios were high only when adult nutrition was high suggests the importance of good nutrition during adult development for proper reproductive function.

4.4. Conclusions

Our study shows that larval nutritional adversity affects adult excretion and that this is mediated by the *for* gene. This demonstrates that early life experience can have lasting consequences into adulthood. Our results suggest that increased *for* expression during chronic stressful larval nutritive conditions may underlie increased excretion output during adulthood. Further study into the observed phenotypes and associated key functional and regulatory tissues such as the gut and trachea will allow us to discover the precise mechanisms underlying this gene-environment interaction. Our results add to the growing body of evidence that early life nutritional experience influences health outcomes later in life. These developmental time periods act and interact in a continuous fashion, not in isolation, on later life history stages (Bagot et al., 2012; Burns et al., 2012; Kristensen et al., 2011; Lin et al., 2013; McDade, 2012). Our approach can be used to investigate how genes and environments interact across development to better understand how early life experiences contribute to phenotypic variation in adult phenotypes.

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