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Bachelor in Biology

# Epigenetics and Behavioural Plasticity:

# Drosophila euchromatin histone metiltransferase and *foraging*

A thesis submitted in fulfillment of the requirements for the degree of Masters in Molecular Genetics and Biomedicine

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“In nature's infinite book of secrecy, A little I can read.”

— William Shakespeare in Antony and Cleopatra.

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# Abstract

The *foraging* gene in *D. melanogaster* underlies a natural polymorphism with two variants called rover and sitter. These variants differ in a suite of phenotypes which are plastic when exposed to varying environmental parameters. Although the phenotypic differences between rovers and sitters are evident, the underlying molecular mechanisms involved are not completely understood. Recently, a histone methyltransferase (*EHMT*) was found to methylate histones at the *foraging* promoter region, suggesting a possible interaction of these two genes. This work provides strong evidence that *EHMT* significantly affects several phenotypic traits linked to the *foraging* gene. *EHMT* is needed for the plastic response to food-deprivation seen in larval feeding behaviour, evident as food-deprived larvae lacking *EHMT* show the same behaviour as fed larva, while larva with functional *EHMT* significantly reduce their path-lengths when food-deprived. Furthermore, the loss of functional *EHMT* affects sitter but not rover adult foraging behaviour in food-deprived flies, suggesting an epigenetic interaction between *EHMT* and the *foraging* alleles. *EHMT* mutants also have higher fat storage levels and survive longer during starvation. And finally, *EHMT* mutants and revertants do not differ significantly in overall *foraging* RNA expression in fed and food-deprived feeding regimes, but show substantial differences in the *foraging* protein isoforms expressed. Taken together, this thesis provides proof that *EHMT* epigenetically regulates traits influenced by the *foraging* gene and that this regulation is linked to environmental cues. Considering that both *foraging* and *EHMT* have homologues in many species and have been associated to a series of human diseases, the results herein are also interesting from a human perspective.

**Key words: behaviour; plasticity; epigenetic regulation; *foraging*; *EHMT***

# Resumo

Em *Drosophila melanogaster*, existem dois variantes fenotípicos associados a um polimorfismo natural com origem no gene *foraging*, nomeadamente “rovers” e “sitters”. Estes variantes diferem num grande número de características fenotípicas e exibem plasticidade comportamental em resposta ao ambiente. Apesar de as diferenças fenotípicas entre “rovers” e “sitters” serem evidentes, os mecanismos moleculares subjacentes não são completamente conhecidos. A recente descoberta que a metil-transferase EHMT metila histonas na zona promotora de *foraging,* sugere uma possível interacção na modulação fenotípica. No presente projecto são apresentadas provas de que o gene *EHMT* influencia significativamente fenótipos associados ao gene *foraging. EHMT* é necessário para a plasticidade no comportamento em resposta à privação de comida uma vez que larvas sem *EHMT* funcional não têm resposta comportamental à ausência de comida. Por outro lado, em adultos a perda de função de *EHMT* afecta o comportamneto alimentar em “sitters” mas não em “rovers”. Além disso, mutantes de *EHMT* acumulam mais reservas de lípidos e sobrevivem mais tempo na ausência de nutrientes do que indivíduos com *EHMT* funcional. Finalmente, os níveis de expressão do gene *foraging* não são significativemente affectados por *EHMT* ou pelo regime alimentar, mas os níveis proteicos das diferentes isoformas codificadas por *foraging* differem substancialmente com a funcionalidade de *EHMT* e com o regime alimentar. Assim, este trabalho apresenta evidências que *EHMT* regula epigenéticamente características controladas pelo gene *foraging* e que esta regulação depende de factores ambientais. Tendo em conta que tanto *foraging* como *EHMT* têm homólogos em muitas espécies e foram associados a uma série de patologias humanas, estes resultados são interessantes numa perspectiva humana.

**Palavras chave: Comportamento; plasticidade; regulação epigenética; *foraging* ;*EHMT***

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# 1 – Introduction

The big question in behavioural genetics is to understand the interrelationship of genetic mechanisms and behavior, studying how genes are regulated throughout the development of an organism and how they give rise to different phenotypes. At the level of the individual, the ability of an organism to change its phenotype in response to variations in the environment is defined as phenotypic plasticity. Between individuals, differences in behaviour originate not only in allelic variation at the genetic level but also in different levels of plasticity in a changing environment. The complex interactions of genes and environment become evident with such differences in behavioural response to environmental parameters.

Questions about how gene-environment interplay (GEI) works (through genotype by environment interactions and/or epigenetic regulation), and how big the role of each of these factors is in modulating behavior are fundamental to biology, psychology and medicine.

The fruit fly *Drosophila melanogaster* (*D. melanogaster*) has been largely used as model organism in both these fields, due to its many advantages. Firstly, *D. melanogaster* is a small species with a short life cycle that can be easily reared in the laboratory to produce large numbers of progeny. Secondly, genetic methods and tools have been developed for *D. melanogaster* to an extent that far exceeds that of any other complex multi-cellular organism. In addition, the high level of homology of genes and cellular pathways between *D. melanogaster* and humans makes the fruit fly an excellent tool for understanding and modeling specific human diseases (Beckingham *et al*, 2005).

A brief description of the *D. melanogaster* life cycle follows below. The developmental period of the fruit fly varies with temperature, ranging from 7 days at 28 °C to over 50 days at 12 °C. At standard laboratory 25ºC and under ideal rearing conditions, the development time is 8.5 days, but under adverse environmental conditions, like crowding or poor food quality, development time increases, while the emerging flies are smaller (Bakker, 1961). At 25 ºC and good rearing conditions, 1$^{st}$ instar larvae hatch 12–15 h after the eggs have been laid and grow for about 4 days while molting twice (into 2$^{nd}$- and 3$^{rd}$-instar larvae), at about 24 h and 48 h after hatching. During this period larvae feed continuously on food substrate at a feeding rate that is age related. The feeding rate increases during the 1$^{st}$ and 2$^{nd}$ larval instars, reaching a maximum during the first half of the 3$^{rd}$ larval instar (Sewell *et al*, 1975 ). This is important because larval feeding behaviour measurably affects the rate of larval development (Sewell and Connolly, 1975), pupation time (Bakker, 1961; 1969) and egg-to-adult viability (Ohnishi, 1979). About 96 h after hatching late 3$^{rd}$ instar larva stop feeding and start

wandering in search of a pupation site. Pupae undergo a four-day-long metamorphosis, after which the adults emerge (Ashburner and Thompson, 1978). The first days after eclosion are critical for cuticle formation and fat storage accumulation and during this period flies are more susceptible to environmental stress (Ashburner, 1989).

## The *D. melanogaster foraging* gene and its pleiotropy

The *D. melanogaster foraging* gene (*for*) encodes a cGMP dependent protein kinase (PKG; Kalderon and Rubin, 1989; Osborne *et al,* 1997), a signaling molecule with varied influences on behaviour and a large degree of pleiotropy (pleiotropy occurs when multiple phenotypic traits are influenced by one gene) and plasticity. *for* is a complex gene that can be linked to a large suite of behavioural phenotypes not only in *D. melanogaster* but in many other species as well (such as the honey bee, ants, nematodes and mammals). The natural behavioural polymorphisms, plasticity and pleiotropy associated to *for* have provided an excellent model for studying how natural variation in a single major gene influences phenotypic traits in diverse taxa, from perspectives as varied as ethology, evolution, genetics and neuropharmacology (Reaume and Sokolowski, 2009).

In the fruit fly *for* has first been associated with larval foraging strategies. In 1980, Sokolowski identified a natural occurring dimorphism in feeding patterns of fruit fly larva. Depending on the distances the larvae travel while foraging they can be divided into two phenotypical groups: either rovers or sitters. While larvae exhibiting the sitter phenotype travel relatively short distances when on a food substrate, rovers show considerably longer path lengths. The expression of this difference in larval locomotory behaviour depends on the availability of food, since on a non-nutritive substrate there is no difference in rover/sitter moving patterns (Sokolowski *et al*, 1983; Kaun, *et al,* 2007a). Genetic analysis using chromosomal substitutions between isogenic stocks, showed that that this behaviour was linked to the second chromosome and that the rover phenotype has complete dominance over the sitter phenotype (deBelle and Sokolowski, 1987). Nevertheless, the rover and sitter phenotypes are both naturally maintained in wild populations, and as several studies showed, populations in the wild have about 70% rovers to 30% sitters (Sokolowski, 1980; 1982; Sokolowski *et al*, 1997). Interestingly, despite the strong association between the rover/sitter *for "*alleles" and the larval feeding patterns, the phenotypes are plastic when exposed to varying environmental parameters. For instance, expression of the larval foraging behaviour was found to be conditional on the availability of food in the environment during larval development, as food-deprived larva showed a considerable reduction in the amount of locomotory behaviour while foraging. Nevertheless, since starvation reduced locomotory rates in both rovers and sitters, the allelic differences in path length were maintained (Graf and Sokolowski, 1989).

Later studies showed that the effects of *for* are not limited to larval foraging behaviour. A correlation between the rover and sitter larval foraging phenotypes and the preference for pupation sites was also found. Although differences in pupation sites couldn't be attributed to *for* alone

(pupation height is a polygenic character influenced by many genes with additive effects on the major autosomes), it was evident that sitters in nature prefer to pupate on their feeding substrate (rotting fruit), while rovers move away from the fruit to pupate. In laboratory vials this difference can also be seen in how high larvae pupate on the vial walls, with sitters pupating preferentially closer to the food than rovers (Sokolowski, 1985). Further investigations of these correlated behavioural traits in rovers and sitters demonstrated that high animal rearing densities selected for the rover phenotype in laboratory populations, while low animal rearing densities selected for the sitter phenotype, this means that density-dependent natural selection produces changes in this trait (Sokolowski *et al*, 1997).

Pereira and Sokolowski (1993) showed that the rover/sitter polymorphism in feeding strategies is maintained beyond the larval stage. *D. melanogaster* rover and sitter strains isolated from nature differ in the distance adult flies walk after feeding per unit time; this is, after feeding on a sucrose drop, rovers move away from the food source, while sitters spend more time circling around the spot. Like in larvae, this variation results from different alleles at the *foraging* locus, evident as mutagenized rover flies carrying a sitter-like *foraging* allele on a rover genetic background (the $for^{s2}$ *foraging* allele) also exhibited sitter behaviour in this adult assay (Pereira and Sokolowski, 1993). Considering that $for^{s2}$ mutant flies have the same genetic background as rovers, differing only in the *foraging* allele, $for^{s2}$ data provides strong evidence that the observed behavioural differences are a function of *for*.

Still in regard to food related traits, it has been shown that rover larvae have lower food intake than sitter and $for^{s2}$ mutant larvae, higher levels of glucose absorption and preferential allocation of glucose to lipids. These differences are dependent on rearing conditions such as food quality or availability. The reduction of quantity and/or quality of the available food results in an overall rise in food intake in both rovers and sitters and rover/sitter differences are lost. Nevertheless, rover larvae maintain higher absorption efficiency but also have more rapid development and higher survivorship compared to sitters and $for^{s2}$ when food is limited and they are grown with their own variants (Kaun *et al*, 2007a).

Furthermore, there are also *foraging* related differences at the neuronal level in *D. melanogaster*. In adult flies, there is an allelic difference in olfactory-related behaviour that can be attributed to the *foraging* rover and sitter alleles. Sitters show a much higher response in the ability to migrate towards the source of a fly medium attractant than rovers and this difference cannot be attributed to general olfactory deficits. Deficiency mapping with $for^{s2}$ mutants revealed that this phenotype originated from the *foraging* locus. This suggests that PKG signaling pathways are involved in olfactory related responses to food (Shaver *et al*, 1998).

Additionally, *for* independently affects sensory responsiveness and habituation in adult flies. An assay, in which the response to sucrose (proboscis extension) was tested, showed that rovers are more responsive to sucrose than sitter and $for^{s2}$ flies, but that this difference decreases with food deprivation. The fact that rovers also show lower habituation indexes under repetitive testing conditions than sitter and $for^{s2}$ flies indicates that *for* also has a function in non-associative learning (Scheiner *et al*, 2004).

Renger *et al* (1999) showed that there also are physiological and morphological variations in nervous systems of the rover and sitter allelic variants isolated from natural populations. Whole-cell

current clamping revealed distinct excitability patterns, with spontaneous activities and excessive evoked firing in sitter, but not rover neurons, as well as reduced voltage-dependent $K^+$ currents in sitter neurons. In addition, sitters show more diffuse motor axon terminal projections with increased ectopic nerve entry points in larval muscles. Data from $for^{S2}$ and two other mutant sitter strains confirmed that these phenotypes are part of *for*'s multiple functions (Renger *et al*, 1999).

More recently, sitters have been shown to have poorer short-term memory but better long-term memory than rovers in an associative olfactory learning paradigm. This difference was linked to expression of *for* in the mushroom bodies evident as by selectively increasing the level of PKG in the mushroom bodies of transgenic sitter flies (with the UAS-GAL4 system), their behavior became rover-like (Mery *et al,* 2007). It had been shown before that the mushroom bodies are central to olfactory learning processes in *D. melanogaster* (Heisenberg, 2003). Olfactory conditioning was also significantly influenced by *for* expression in the mushroom bodies of larvae, where rovers showed faster memory acquisition and longer retention than sitters and $for^{s2}$ (Kaun *et al*, 2007b).

Finally, the natural foraging variants also differ in their response to environmental stress, such as heat or anoxic conditions. Sitters are considerably more thermotolerant, maintaining normal synaptic transmission at significantly higher temperatures than rovers. Pharmacological manipulations of the PKG pathway showed that this was directly related to PKG activity (Dawson-Scully *et al*, 2007). The same seems to be the case with hypoxia (Reaume and Sokolowski, 2009). In addition, sitters survive longer when there is no food available (Donlea *et al*, 2012), this could be related to the fact that sitters have higher fat storage levels than rovers (Kent *et al*, 2009).

Considering all of the above, the *D. melanogaster foraging* gene presents an excellent model to study GEIs.

## Molecular structure and expression patterns of *foraging*

Although the phenotypic differences between rovers and sitters are evident, so far it is not clear from where these differences originate at a molecular level. There has been identified a large number of polymorphisms in the nucleotide sequence of the natural *for* allelic variants, but none have been directly linked to the plasticity observed in the rover/sitter phenotypes (Aaron Allen, personal communication).

As mentioned above, *foraging* encodes for one of two cGMP-dependent protein kinases (PKG) in *D. melanogaster* (*dg1* and *for/dg2*), PKGs are signaling molecules that act as key mediators of the nitric oxide (NO)/cGMP signaling pathway by phosphorylating serines and threonines on many cellular proteins. Unfortunately it is still unclear how exactly PKG acts at a cellular level since it remains mostly unknown what genes and molecules are involved up and downstream in the PKG signaling cascade.

Nevertheless, there is extensive data on *for* expression patterns available on fly base (FlyBase, 2012). Overall expression of *foraging* is highest in early embryogenesis (0 - 4 h old embryos), after that, expression decreases to a moderate level when the embryos are about 12 h old and increases

again to higher level until the end of embryogenesis. Lowest expression levels, are observed in L1, L2 and early L3 larvae. In late L3 larvae expression increases again and is maintained during the first 24 h of pupation. In the last 24 h of pupation, expression decreases to L1/L2 larva levels. In adults, there is a sex difference in expression levels, while males only show moderate overall expression, females show levels twice as high (modENCODE Temporal Expression Data; Graveley *et al*, 2011). In more detail, in L3 larva the highest *for* expression is found in the fat body and trachea, lower but still high expression levels are seen in the midgut, hindgut and salivary glands, and moderate expression is found in the central nervous system and the malpighian tubules. In Adults, the highest expression levels are found in female spermatheca, but levels are also very high in the fat body, ovaries, crop and head. Moderate levels are found in tissues of the digestive tract (FlyAtlas Anatomical Expression Data; Chintapalli *et al*, 2007). In summary, *for* is expressed at all developmental stages and in all analyzed tissues, but the temporal and spatial distribution varies greatly.

Structurally, the *foraging* gene comprises about 35000 base pairs and is located on the long arm of chromosome 2 (deBelle *et al,* 1989). There are 11 annotated transcripts of *for* that code for 11 annotated PKG isoforms, transcribed by 4 known promoters (FlyBase, 2012), but unpublished data from the Sokolowski Lab (Aaron Allen, personal communication) shows evidence for at least another 10 transcripts, all with open reading frames. If the different isoforms of PKG vary in their functions, and how their expression varies across development stage and tissues, remains mostly unknown.

Interestingly, Osborne *et al* (1997) showed that rovers and sitters differ in PKG activity levels. Rover larvae showed higher *for* RNA expression (for the 3 transcripts known at the time), as well as higher PKG protein levels and higher PKG activity than sitter larvae. Since transgenic sitters expressing a *for* complementary DNA from rover showed transformation of larval foraging behaviour to rover type, the behavioural polymorphism observed in food search can be linked to natural variation in PKG activity. The same was the case for adult flies, were *for* RNA levels, PKG protein levels and PKG activity were higher in rover heads than in sitter heads (Osborne *et al*, 1997). Immunohistochemical analyses with a *for*-specific antibody showed that FORAGING (FOR) localizes to neurons in the adult brain as well as to the antennal nerve, which carries axons of the olfactory, auditory, and mechanosensory neurons. In addition, FOR expression in the optic lobes suggested a function in reception and/or processing of visual stimuli. However, rovers and sitters did not show obvious differences in FOR spatial distribution (Belay *et al*, 2007). It is possible that the rover-sitter differences observed in neuronal behaviours depend on expression levels of a specific FOR isoform, or expression at a specific developmental stage.

## Importance of *foraging* in other species

As mentioned earlier, homologs of *for* exist across many other species where they are associated to a variety of behaviors and molecular functions. In the honey bee, expression levels of the *for* homolog *Amfor* play a role in phototaxis and seem to determine the transition from nursing behaviour to foraging behaviour, thus defining division of labour in bee hives (Ben-Shahar *et al,* 2002;

2003). In harvester ants, task-specific expression levels of *Pbfor* also determine colony organization by influencing foraging behaviour, and mediating the switch between foraging and defense behaviour (Ingram *et al*, 2005; Lucas and Sokolowski, 2008). In *C.elegans*, the *for* homolog *egl-4* has been found to play a role in sensory neurons for modulation of sensory information and to regulate growth and locomotory behaviour (Fujiwara *et al*, 2002; L'Etoile *et al*, 2002). In mammals, cGMP-dependent protein kinases are expressed in many different tissues including smooth muscle, platelets, intestine, and brain (Pfeifer *et al*, 1999). More specifically, in mice, cGMP signaling is important for axonal growth (Schmidt *et al*, 2002), plays a role in nociceptive transmission in the spinal cord (Tegeder et al, 2004) and is involved in the regulation of cocaine-related effects on behaviour (Jouvert *et al*, 2004). Furthermore cGMP-dependent protein kinase I (cGKI) is required for cerebellar long-term depression (lasting activity-dependent reduction in the efficacy of neuronal synapses) and specific forms of motor learning (Feil *et al*, 2003), as well as age- and protein synthesis-dependent hippocampal long-term potentiation (long-lasting enhancement in signal transmission between neurons; Kleppisch *et al*, 2003).

The human homologue of *for* (*PRGK1*) has also been associated with a series of metabolic pathways and functions. In inflammatory immune response, protein levels of Interleukin 6 are dependent on the concentration of components of the cGMP/PKG pathway (Siednienko *et al*, 2011). Also, PRKG1 seems to be involved in endothelial dysfunction and other vascular diseases, being a regulator of blood pressure and vascular tone in endothelial and smooth muscle cells (Gebska *et al,* 2008; Tang *et al*, 2003 ). In addition, as one of the main receptors for cGMP, PKGs mediate most of the effects of cGMP elevating drugs, such as nitric oxide-releasing agents and phosphodiesterase inhibitors which are used for the treatment of angina pectoris and erectile dysfunction, respectively (Kim *et al,* 2011). Polymorphisms in *PRKG1* have been found to be associated with Type 2 diabetes (Saxena *et al,* 2007).

## Epigenetic modulation of gene expression in *Drosophila*

Considering all that is known about the *foraging* gene and its role in behavioural phenotypes and physiological processes, one of the big questions that come to mind is how this gene is regulated. Over the past few years researchers in many fields have progressively focused on understanding differences in gene expression that are not mediated at the DNA sequence level. Examples of such mechanisms underlying the differential expression of genes are DNA methylation and histone modifications. Stable alterations of this kind are said to be 'epigenetic', because they are heritable in the short term but do not involve mutations of the DNA itself (Cheung and Lau, 2005). Epigenetic processes are important for development, but they can also arise in mature organisms, either by random change or under influence of the environment (Jaenish and Bird, 2003).

Until recently, epigenetic research has been primarily focused on mammalian model systems, and although DNA methylation has been described in several other insect species (Field *et al*, 2004),

researchers had failed to detect methylated bases in the *D. melanogaster* genome. Several studies reporting the complete absence of DNA methylation in fly embryos (Urieli-Shoval *et al*, 1982), pupae (Patel and Gopinathan, 1987) and adults (Bird and Taggart, 1980; Rae and Steele, 1979) have led to the assumption that the fruit fly belongs to an atypical group of animals with no detectable genomic DNA methylation (Bird, 1995). This was countered by the discovery of cytosine methylation in early development stages of fly embryos by the eukaryotic DNA methyltransferase *Dnmt2,* the single candidate DNA methyltransferase gene in the *Drosophila* genome (Gowher *et al*, 2000; Lyko *et al*, 2000).

But epigenetic regulation is not mediated by DNA methylation alone. Other important factors are the methylation and acetylation of nucleosome histones, processes that are major determinants of chromatin structure and gene expression. On one hand, histone acetylation directly affects the condensation state of the DNA by removing the positive charge on the histones, and thereby decreasing the interaction of the N termini of histones with the negatively charged phosphate groups of DNA. On the other hand, methylation marks act as general signaling platforms, by specifically recruiting effector proteins to characteristic landmarks along the DNA.

Contrary to DNA methylation, histone modifications are a well-known phenomenon in *D. melanogaster* (for review see Boros, 2012) and recently the *Drosophila euchromatin histone methyltransferase* (*EHMT*), a member of a conserved protein family that methylates histone 3 at lysine 9 (H3K9), was found to methylate histones in the region of the *foraging* gene promoters (Kramer *et al*, 2011).

Methylation of H3K9 is generally associated with formation of heterochromatin and consequently gene repression (Bannister *et al,* 2001; Jacobs *et al*, 2001; Lachner *et al,* 2001). Besides *EHMT*, there are two other known H3K9-specific HMTases in *Drosophila* that have been well characterized. *SU(VAR)3-9* was shown to di- and trimethylate H3K9 at the chromocenter (Schotta *et al,* 2002; Ebert *et al,* 2004) and *DmSETDB1* mono- and dimethylates H3K9 in euchromatin, dimethylates H3K9 on chromosome 4, and is required for silencing of variegating transgenes on chromosome 4 (Seum *et al*, 2007). *EHMT* was first identified by Stabell et al (2006), where it was described as the *Drosophila* homolog of the mammalian *G9a*, a histone methyltransferase that mono- and di-methylates H3K9 at euchromatic loci. *In vitro, EHMT* specifically mono-, di- and trimethylates lysines 9 and 27 at histone 3 and lysine 8, 12 or 16 in histone 4, a methylation pattern that is mainly correlated with gene silencing in the euchromatic region of the DNA (Martin and Zhang, 2005). Stabel *et al* reported that *dG9a* (*EHMT*) RNA was present in low amounts in 0–3 h old embryos, but with these transcripts probably being from maternal origin, since in 3–6 h old embryos the expression of *EHMT* was barely detectable. In late embryogenesis and throughout larval development the expression of *EHMT* was low but clearly discernible, with a slightly elevated expression during the third larval instar. There were no transcripts detectable in pupae, and in adult flies the expression was restricted to the gonads in both sexes. Interestingly, *EHMT* protein seems to accumulate in the ovary nurse cells, from where it is dumped into the growing oocyte, which indicates a role for *EHMT* in germ cell formation. Furthermore, the results suggested that *EHMT* is required for normal development, since RNAi knock out flies failed to undergo the transition from 3$^{rd}$ instar larvae to pupation. A role for

*EHMT* in regulation of genes correlated to ecdysone responsive signaling, especially during the onset of metamorphosis and wing development was therefore proposed (Stabell *et al,* 2006).

Contrary to these results, Seum *et al* (2007) described *EHMT* as being a non-essential gene that is not required for fly viability. They reported homozygous deletion mutants to be viable and fertile, with no particular phenotype and could not find any *EHMT* mediated H3K9 methyltransferase activity *in vivo* (Seum *et al,* 2007).

Recently, Kramer *et al* (2011) dissected the neuronal function of *EHMT* and found that *EHMT* mutants are viable and develop normally, but that *EHMT* regulates specific aspects of neuronal development and function. The study provided evidence that *EHMT* is widely expressed in the central nervous system and that it induces H3K9 dimethylation at about 5% of the euchromatic genome. The loss of *EHMT* in null mutants resulted in a decrease in dendrite branching in sensory neurons of the peripheral nervous system and in altered locomotory behaviour, as well as impaired non-associative learning and short- and long-term memory. Interestingly, many of the genes involved in the non-associative learning process are involved in cAMP and cGMP second messenger signaling pathways (Engel and Wu, 2009) and the fact that memory could be restored upon re-expression of *EHMT* in the nervous system of the adults suggests that *EHMT* mediates a dynamic epigenetic regulation in neurons (Kramer *et al*, 2010).

The relationship between transcriptional plasticity mediated by *EHMT* and behavioural phenotypes is also interesting from a human perspective, since mammals have two known *EHMT* paralogs (*EHMT1/GLP* and *EHMT2/G9a*) that form a heterodimeric complex which, like in flies, mediates gene repression by H3K9 dimethylation (Tachibana *et al*, 2005). In mice *EHMT* is essential for early embryogenesis and functional mutations in *EHMT* have been associated with severe growth retardation and early lethality (Tachibana *et al*, 2002) as well as cocaine addiction (Maze *et al*, 2010). In humans loss of function mutations in *EHMT* are one of the causes of Kleefstra Syndrome, a neurodevelopmental disorder that is characterized by autistic-like features and severe intellectual disability (Kleefstra *et al,* 2006). In addition, *EHMT* targets include fly orthologs of many genes underlying syndromic and non-syndromic forms of intellectual disability (Kramer *et al,* 2011).

## The relationship of *EHMT* and *foraging*

Since the interplay between genes, transcriptional regulation, behaviours and environment is still poorly understood, it is important to find models that permit the study of these interactions. Taking into account the functional significance of *for* and *EHMT* and the fact that *EHMT* methylates histones in the *for* promoter region (evident as methylation at the *for* promoter is lost in *EHMT* null mutants), as well as the pleiotropy and multitude of physiological processes and phenotypes associated with *EHMT* and *for*, these two genes provide an excellent model to study both the genetic and environmental contributions to individual variations in animal behaviour.

With this project we aimed to shed light on how *for* is regulated and what mechanism(s) underlie the phenotypic differences between rovers and sitters, as well as test the hypothesis that epigenetic

regulation mediates plasticity in response to changing environmental conditions. For this, we started to tease apart interactions between *EHMT* and *for*, working with double mutants of these genes and performing a series of behavioural and molecular tests.

Results showed that the loss of *EHMT* significantly affects several phenotypic traits linked to *foraging*. *EHMT* mutants showed higher fat storage levels and survived longer during starvation. In addition, food related movement patterns in larva and adults were affected. Interestingly, the effect of *EHMT* (or the loss of it) does not always seem to affect rovers and sitters equally, and in a general way, sitters seem to be more affected than rovers. Moreover, in some cases, the effect of the loss of *EHMT* only became evident by altering environmental parameters like food availability, suggesting that the epigenetic regulation of *foraging*, mediated by *EHMT*, might be linked to environmental cues.

# 2 - Materials and Methods

## 2.1 - Fly Stocks

The *D. melanogaster* rover and sitter lines were obtained from Sokolowski Lab central stocks maintained at the University of Toronto, Canada. $EHMT^{+}$, $EHMT^{DD1}$, $EHMT^{+};for^{R}/CyO$, $EHMT^{+};for^{S}/CyO$, $EHMT^{+};for^{s2}/CyO$, $EHMT^{DD1};for^{R}/CyO$, $EHMT^{DD1};for/CyO^{S}$ and $EHMT^{DD1};for^{s2}/CyO$ lines were kindly provided by Jamie Kramer (University of Nijmegen, Netherlands).

The homozygous rover and sitter lines originated from wild type stocks that were selected for the $for^{R}$ and $for^{S}$ alleles based on rover or sitter phenotypes in larva path length assays (de Belle and Sokolowski, 1987). These lines have been repeatedly tested over many generations to ensure they maintain the rover and sitter larval foraging phenotypes.

The $for^{s2}$ line is a mutant line consisting of a sitter allele in a $for^{R}$ second and third chromosome genetic background (Pereira and Sokolowski, 1993), and a simplified description of how it was generated is briefly described below. Homozygous $for^{R}$ males were exposed to gamma irradiation and subsequently crossed to homozygous $for^{S}$ females. Since $for^{R}$ is dominant over $for^{S}$, F1 individuals expressing a sitter phenotype were selected to screen for mutations in the foraging gene (deBelle *et al*, 1989). The resulting $for^{s2}$ line is homozygous for a mutation in *for* which results in sitter larval behaviour on a rover genetic background.

$EHMT^{DD1}$ and $EHMT^{+}$ are P-element excision lines, derived from a reversion event where element KG01242 was inserted in the 5' UTR of the *EHMT* gene. $EHMT^{DD1}$ was obtained by imperfect KG01242 excision, resulting in a 870 basepair deletion downstream of the original P-element insertion site, which includes the *EHMT* translational start site. $EHMT^{+}$ served as a control in all experiments and is a precise transposon excision line that represents the same genetic background as the deletion line. The original P-element line had an $y^{1}$ mutated background, this is, a loss of function mutation in the *yellow* gene that results in defect body pigmentation, where mutant flies exhibit lighter body pigmentation than wild type flies. This background permitted detection of the P-element that has a functional *yellow* allele. Since *yellow* segregates with *EHMT*, after successful excision of *KG01242*, all excision lines show the $y^{1}$ phenotype. Absence of functional and truncated *EHMT* in the $EHMT^{DD1}$ line and presence of functional *EHMT* in the $EHMT^{+}$ line have been previously confirmed by western blot

and immunohistochemistry analysis. Importantly, the neighbor gene, *CG3038*, was not affected by the deletion; consequently, $EHMT^{DD1}$ is a strong and specific loss of function mutant and most likely a complete null allele (Kramer *et al*, 2011).

Initially, we obtained lines from Jamie Kramer which were made by crossing the $EHMT^{DD1}$, $EHMT^{+}$, $for^{R}$, $for^{s}$ and $for^{s2}$ lines described above. These lines ($EHMT^{+};for^{R}/CyO$, $EHMT^{+};for^{s}/CyO$, $EHMT^{+};for^{s2}/CyO$, $EHMT^{DD1};for^{R}/CyO$, $EHMT^{DD1};for^{s}/CyO$ and $EHMT^{DD1};for^{s2}/CyO$ ) are homozygous for the X chromosome with a $y^{1}$ mutation and have either the $EHMT^{DD1}$or $EHMT^{+}$ allele; heterozygous for the second chromosome from $for^{R}$ , for$^{s}$ or $for^{s2}$ lines and a *CyO* balancer chromosome. To obtain homozygous *for* lines, heterozygotes were crossed and *CyO* phenotypical marker (curly wings) selected against. The resulting lines were therefore homozygous for the X and 2$^{nd}$ chromosome and uncontrolled for the 3$^{rd}$ and 4$^{th}$ chromosomes. Since the $EHMT^{DD1};for^{R}$ is not viable as a homozygous population, this stock was maintained using the 2$^{nd}$ chromosome balancer *CyO* ($EHMT^{DD1};for^{R}/CyO$). These heterozygous populations originate 25 % healthy but sterile homozygous $EHMT^{DD1};for^{R}$ progeny each generation and only homozygous flies were used as test animals. The genotypes used for all subsequent tests were therefore: $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$.

## 2.2 –Stock Maintenance and Starvation Treatments

Stock populations were kept on standard Sokolowski Lab medium (see below) at 20 ºC and 40 - 60 % humidity with a 12 h light/dark cycle with lights on at 0800 h. Stock populations were changed into new vials every 15 days. Test populations were reared on standard Sokolowski Lab medium at 25 ºC and 60 % humidity with a 12 h light/dark cycle with lights on at 0800 h. The standard fly medium was prepared by mixing an autoclaved solution A (200 g sucrose, 16 g agar, 1 g $KH_2PO_4$, 8 g KNa Tartrate, 0.5 g NaCl, 0.5 g $CaCl_2$, 0.5 g $MgCl_2$, and $Fe_2(SO_4)_3$ in 800 mL of $dH_2O$) with solution B (25 g of dried yeast in 200 mL of $dH_2O$ at 50 ºC) and adding 5 mL of propionic acid. Either 60 mL polystyrene fly vials with matching buzzplugs and 10 mL of fly medium or 60 oz plastic culture bottles with matching buzzplugs and 25 mL of standard medium were used.

For food deprivation, fly vials were prepared with 10 mL of 1 % agar. A 2 mL eppendorf tube, filled with tap water and capped with a cotton disc, was embedded in the agar; this provided the flies with water to reduce any exacerbating effects of dehydration on the starvation stress. Food deprivation vials were stored at 4 ºC under airtight conditions for no more than 2 days.

To select first instar larva for behavioural experiments, grape juice agar was used, as the dark purple color medium provides increased contrast for picking larvae from the surface. The grape juice agar was prepared bringing 1.8 g of agar in 50 mL of $dH_2O$ and 45 mL of purple grape juice to a boil, cooling to 55 ºC and adding 2.5 mL acetate and 2.5 mL ethanol to prevent fungus from forming on the plates. The lids of 35x10 mm petri plates (these 35X10 mm lids fit perfectly into the holding bottles used in larval experiments) were filled to the rim with grape juice agar and stored at 4 ºC. To obtain

eggs, grape plates were fitted in the top opening of 60 oz plastic bottles with a hole cut in the side plugged with a sponge for air circulation. Once selected, first instar larva were placed in 100x15 mm petri dishes with 35 mL of standard medium for fed conditions and 35 mL of 1 % agar for food deprived conditions and allowed to develop under standard conditions.

## 2.3 – Viability Test

Viability tests were performed on $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$ and $for^{R}$ , $for^{s}$ and $for^{s2}$ strains. To control for larval density and ensure a healthy feeding environment, parental populations were set up using 5-6 days old flies. 15 virgin females and 8 males were introduced into standard rearing vials and individuals eclosed from these vials tested for viability. Once eclosed, 15 virgin females and 5 males were collected using $CO_2$ anesthesia and aged separately for 5 - 6 days in fresh vials. After 5 - 6 days, males and female flies were placed together in holding bottles with grape plates containing yeast paste (pure yeast and water) to increase egg production. These populations were given an acclimatization period of 48 h, during which the grape plates were replaced every 24 h. After 48 h new grape plates were introduced and females were allowed to lay eggs for 4 h. Eggs were counted and 100 eggs were transferred on to 100 x 15 mm petri dishes with standard fly medium. L1 larva were counted after 24 and 32 h and introduced into rearing vials, in groups of 20 larvae. After 168 h and 10 days the number of pupae and the number of eclosed flies was scored. The animals were kept at 25 ºC and 60 % humidity with a 12 h light/dark cycle at all times and the test was repeated 5 times.

## 2.4 – Sterility Test

To further analyze the sterility $EHMT^{DD1};for^{R}$ strain, homozygous $EHMT^{DD1};for^{R}$ were crossed to wild type rover flies, where 15 virgin $EHMT^{DD1};for^{R}$ females and 5 $EHMT^{DD1};for^{R}$ males were crossed to 5 wild type $for^{R}$ males and 15 virgin $for^{R}$ females, respectively. The flies were allowed to lay eggs for 24 h on newly introduced grape plates, and the plates containing eggs incubated without flies for another 24 h. After this time, grape plates were screened for the presence of larva every 24 h for 2 days. The animals were kept at 25 ºC and 60 % humidity with a 12 h light/dark cycle at all times and the test was repeated 5 times.

## 2.5 – Larval Path Length Test

For the larval path length test, populations were built up in bottles for 3 generations. At this time, the eclosed progeny of the 3rd generation was collected over 3 days. Flies were then aged for 5 - 6 days before setting up the experiment. To obtain test larvae, these flies were transferred into holding bottles with grape plates to which a dab of thick yeast paste had been added in order to increase egg production. These bottles were incubated for an initial period of 24 h with grape plates facing down to ensure the flies were acclimatized to their new environment, at which time the plates replaced and incubated with flies for a further 24 h. After this 48 h, flies were removed and plates incubated for 20 h before clearing all eclosed larvae. For collection of precisely staged larvae, cleared plates were incubated for 4 h and test larvae collected.

Due to the large number of strain-treatment combinations tested, and the importance of exact timing, the strains were randomly divided into 3 test groups. In test group 1, a fresh grape plate was placed in the holding bottles at 1:00 pm; whereas fresh grape plates were added to bottles in test group 2 and 3 at 2 pm and 3 pm, respectively. As described above, flies were allowed to lay eggs on these grape plates for 24 h at which time grape plates were substituted and flies allowed to lay eggs for another 24 h. The grape plates with eggs were incubated for 20 h and cleared of all larvae using a probe under the microscope (i.e. test group 1, 2 and 3 were cleared of larva at 9, 10 and 11 am, respectively). The cleared plates were incubated for a further 4 h and first instar larva picked and placed on Petri dishes containing 35 mL of Drosophila medium (100 larvae were placed on each plate to control for density). Test group 1, 2 and 3 larvae were collected at 1, 2 and 3 pm, respectively. To obtain 3rd instar larva, plates were incubated for 92 h and 80 late 3rd instar larva (92 ± 0.5 h old) were then picked, rinsed with water and divided into 2 groups. The first group was placed into 100 x 15 mm petri dishes with 2 circular 50 mm diameter nutrient medium discs, the second group was food deprived in 100 x 15 mm petri dishes with 2 circular 50 mm diameter 1 % agar discs. Larva were incubated for another 4 h and then tested. Animals were kept at 25 ºC and 60 % humidity with a 12 h light/dark cycle at all times.

The larval foraging path length assay (Pereira *et al*, 1995) was developed to assess rover-sitter movement differences of *Drosophila* larva during foraging. The test measures the distance an individual larva travels during foraging over a 5 min period. The set up for this assay consists of plexiglass plates thinly spread with a homogeneous yeast suspension made of distilled water and Fleischmann's bakers' yeast in a 2:1 ratio by weight. This yeast paste permits to see the path a larva travelled during the test period. Each black plexiglas plate (25 cm width, 57 cm length, 0.5 cm height) has ten 0.5 mm deep circular wells of 8.5 cm diameter arranged in a 2 X 5 fashion. This set up permits the testing of a large number of larvae simultaneously.

For behavioural testing, larvae were gently picked from the food or agar using a small paintbrush, rinsed with $dH_2O$ to remove remaining food particles, and individual larvae placed in the center of the yeast coated wells. The wells were covered with a transparent plastic petri dish lid to prevent the drying out of the yeast paste and stop the larvae from leaving the test area. The testing period started from the moment the first larva was placed on the plate and ended after 5 min. Path

lengths were traced on the lids using a marker and the lids carefully kept for digitalization and statistical analyses. Following data collection, the path lengths recorded on the petri dish lids were passed onto white paper sheets using a lighted tracing table and scanned into the computer. Path lengths were measured with Image J 1.45 utilizing a 1 cm measured line as scale, therefore providing the path length in cm for each larva tested. Two separate experiments were conducted with this assay, as detailed below.

In the first experiment, $EHMT^{+}$ and $EHMT^{DD1}$ strains were tested with $for^{R}$ and $for^{s}$ as standard laboratory control lines. For each strain, 40 fed and 40, 4 h food deprived larva, exactly 96 ± 0.5 hours old, were tested individually. To assess male-female differences, the tested larvae were introduced into numbered isolation vials after being tested. The isolation vials were incubated at 25 ºC and 60 % humidity with a 12 h light/dark cycle and flies were scored for sex after eclosion. The test was repeated over 3 consecutive days.

In the second experiment, the following strains were tested; $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$ as well as standard laboratory control lines $for^{R}$ , $for^{s}$ $for^{s2}$. The procedure followed was the same as in the previous experiment, although larvae were not scored for sex. For $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$, $for^{R}$ , $for^{s}$ and $for^{s2}$ 30 larvae were tested. However, since the $EHMT^{DD1};for^{R}$ strain was maintained as $EHMT^{DD1};for^{R}$/CyO, but only individuals homozygous for the 2nd chromosome could be used in the analyses, 90 larva were tested for this strain (considering an expected ratio of 1:3 $EHMT^{DD1};for^{R}/CyO$ progeny to be homozygous) and placed into isolation vials after testing (12x55 mm glass vials with 1 mL of standard medium). These flies where reared to adulthood and screened for the curly wings phenotypical marker and only the larvae path length data from flies with wild type wings (therefore homozygous for $for^{R}$) were used for analysis. This experiment was repeated over 5 consecutive days.

## 2.6 – Adult Foraging Assay

This assay was developed by Bryon Hughson in the Sokolowski Lab to screen for rover/sitter differences in adult feeding behaviour. The procedure was based and modified from previously used adult locomotory assay (Pereira and Sokolowski, 1993). Strains tested in this assay were $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$ and $for^{R}$, $for^{s}$ and $for^{s2}$ as standard laboratory control lines. All tests were made under constant 23 - 24 ºC with 30 - 40 % humidity, during 1-5 pm to control for the effects of circadian feeding patterns. Tests were repeated over 10 days and sample sizes of n = 30 were tested for each strain. Strains were randomized and testing was performed without knowledge of the strains being tested.

To control for larval density and ensure a healthy feeding environment, parental populations were set up using 5 - 6 days old flies. 15 virgin females and 8 males were introduced into standard

rearing vials and 5 vials were set up for each genotype. The flies were allowed to lay eggs for 4 - 5 days and then brooded over into new rearing vials. After being brooded over, flies were given another 4 - 5 days to lay eggs and were then discarded. The vials with the larva were reared to adulthood and these adults were used as test animals. Test animals were collected on the first 3 days of eclosion using $CO_2$ anesthesia and transferred into new rearing vials, individuals with obvious developmental abnormalities being discarded. The reason for collecting flies from the first three days only is that from the $4^{th}$ day of eclosions onward, undersized flies appear. Because of the influence of body size (and correlation with macronutrient reserve quantities contained within the fat bodies) on feeding-related phenotypes these later eclosing undersized individuals were never used as test animals or as parents. Once eclosed, virgins females and males were aged for 5 - 6 days in nutrient vials (n = 15 - 20 / vial), and transferred to fresh vials every day, to ensure prime food quality. 24 h after collection, the wings of the females were clipped to keep the test flies from jumping onto the lid of the arena during the assay. For wing clipping, females were removed from the rearing vials with an aspirator and anesthetized on a $CO_2$ pad. To minimize $CO_2$ exposure and reduce possible effects on behaviour, flies were anesthetized and clipped one at a time, taking less than 10 seconds for each fly. The wings were clipped of at the base using dissecting scissors and tweezers under a microscope. After wing clipping, flies were introduced back into the nutrient vials. 48 h before the start of the test males and females of each genotype were mixed and allowed to mate. All test flies were food deprived 24 ± 0.5 h before being tested, using the food deprivation vials described in 2.2. Only females were tested in this assay.

The set up for this assay consisted in a 15 cm diameter x 1.5 cm height round arena with an acclimatization chamber underneath and an elevator shaft through which the test animal was allowed to enter the arena at the center. The arena was divided into 4 quadrants, each with a 2 cm circle in the center. In the center of each circle was placed a 0.2 µL drop of 0.1 % sucrose with blue food dye. The rim around the edge of the arena was filled with water to prevent the flies from climbing up the walls and a lid was placed on top of the arena that allowed for uniform illumination.

For testing, a single fly was introduced into the acclimatization chamber underneath the arena using an aspirator, and given a 2 min acclimatization period. After this period the barrier to the elevator shaft was removed and the fly was allowed to climb into the arena. Special care was taken to not disturb the fly during this process. The test period started at the moment the fly entered the arena, and the number of sucrose drops the fly consumed was scored at 5 and at 10 min.

## 2.7 – Starvation Resistance Assay

The starvation resistance assay aims to assess differences in survivorship of adult flies in an environment with no nutrient availability. Flies were reared and tested at 25 ºC and 60 % humidity in a 12:12 L:D cycle at all times and incubators were not opened except for scoring to minimize disturbances that could cause stress to the test animals. Strains tested in this assay were $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$ and $for^{R}$, $for^{s}$ and $for^{s2}$ as standard laboratory control lines.

For this assay, the parental generation was reared as in 2.6. Test animals were collected on the first 3 days of eclosion using $CO_2$ anesthesia and aged for 5 - 6 days in nutrient vials with an n = 20 in each vial. Test animals were changed over to fresh nutrient vials every 24 h, and 48 h before the 10 test females in each vial were allowed to mate with 10 males (these males were not used in the test). Males and females were tested separately in this assay. 10 males or females were introduced into food deprivation vials described in 2.2 using an aspirator and no anesthesia. 10 replicate vials were set up for females and males of each strain and replicates were set up at the same time (t = 12 pm ± 0.5 h) to eliminate day and circadian feeding pattern effects.

During the test period, all vials were scored for dead flies every 6 h (i.e. at 12 pm, 6 pm, 12 am and 6 am). The test lasted until the last fly was dead and a fly was scored as dead if motionless even upon light tapping of the vial.

## 2.8 – Total Triglyceride and Protein Quantification

Total triglyceride levels were measured in female flies to assess differences in fat storage in the test strains. For this purpose, total protein levels were used as a reference to eliminate possible variations during the extraction process. Parental generations were set up as described in 2.6 and 6 vials were set up for each of the following strains: $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$ and $for^{R}$, $for^{s}$ and $for^{s2}$ as standard laboratory control lines. To control for density and assure equal feeding conditions, test females were collected over the first 3 days of eclosion and transferred into fresh nutrient vials with an n = 20 per vial,. Flies were allowed to age 4 - 5 days and test flies were transferred into fresh nutrient vials every 24 h to guarantee food quality. After this time, 5 males were introduced in the vials and the females were allowed do mate for 24 h (males were not tested in this assay). 5 - 6 day old flies were separated into 2 experimental groups, one group was maintained on standard rearing nutrient medium for 24 h and the second group was transferred into food deprivation vials described in 2.2 and food deprived for 24 h. Flies were maintained at 25 ºC and 60 % humidity in a 12:12 L:D cycle at all times. Fed and food deprived flies were anesthetized with $CO_2$ and groups of 4 flies were transferred into previously weighted 1.5 mL eppendorf tubes. The tubes with the flies were weighted and flash frozen in liquid nitrogen. Samples were stored at -80 ºC until further use.

Total triglyceride and protein homogenates were prepared as previously published (Grönke, et al., 2005) with the modifications described below. Sample tubes with flies were removed from -80 ºC and processed on ice as follows: 500 µL of 0.5 % Tween in PBS buffer was added to each sample, samples were homogenized for 30 sec with a mechanical homogenizer (or until no noticeable chunks of tissue were left), another 500 µL of 0.5 % Tween in PBS was added and samples were vortexed for 5 sec. After vortexing, samples were incubated in a 70 ºC water bath for 5 min and cooled on ice for 2 min. Samples were centrifuged for 1 min at 5000 rpm and 500 µL of the supernatant was transferred into new 1.5 mL eppendorf tubes. Finally, samples were centrifuged for 3 min at 14000 rpm and 450

µL of the supernatant was transferred into new eppendorf tubes and stored at -80 ºC until further use. 10 biological replicates were made for each strain.

To quantify the concentration of triglycerides in the homogenates the Triglyceride reagent (TAG) from Sigma-Aldrich (cat#TR0100) was used. This reagent permits the quantitative enzymatic measurement of glycerol, true triglycerides and total triglycerides. The procedure involves enzymatic (lipase) hydrolysis of the triglycerides to glycerol and free fatty acids. The glycerol produced is then measured by coupled enzyme reactions resulting in the production of a quinoneimine dye that shows an absorbance maximum at 540 nm which is directly proportional to triglyceride concentration of the sample. The procedure followed was modified from the manufacturers' instructions: triglyceride standards were prepared using the 200 mg / mL mixed triglyceride standard from Sigma-Aldrich (cat#17811-1AMP). To calculate a standard curve, 6 standard dilutions were prepared to the final concentrations of 0, 0.025, 0.05, 0.1, 0.2 and 0.4 mg / mL. The standards were diluted in 0.5 % Tween in PBS buffer, incubated in a 70 ºC water bath for 5 min and cooled on ice for 2 min. For blank absorbance readings, 3 technical replicates with 50 µL of sample and standard dilutions were transferred into 96 well plates and absorbance was measured at 540 nm using the Synergy HT spectrophotometer from BioTek Instruments. After the blank reading, 200 µL of TAG reagent preheated to 37 ºC was added to each well, the plates were incubated for 5 min at 37 ºC and another reading at 540 nm performed. The software used for procedure setup and data acquisition was Gen5 1.10 from BioTek.

The blank absorbance values of each replicate were subtracted from the 5 min incubation absorbance value and the mean value for the 3 technical replicates of each sample was calculated. A standard curve was traced plotting the absorbance values of the 6 standard concentrations against their respective concentrations. The standard concentration with the absorbance value closest to the sample absorbance values was used to calculate the sample concentrations. Sample concentrations were calculated according to $[sample] = \frac{A(sample)}{A(standard)} * [standard]$.

The same samples were tested for total protein content using the Pierce BCA Protein Assay Kit from Thermo Scientific (cat#23227). This assay is based on a detergent-compatible reaction with bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. The method used in the assay combines reduction of $Cu^{+2}$ to $Cu^{+1}$ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation ($Cu^{+1}$). The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a working range of 20-2000 µg / mL. The procedure followed was modified from the manufacturers' instructions: standards were prepared using the 200 mg / mL bovine serum albumin (BSA) provided with the kit. To calculate the standard curve, 6 standard dilutions of BSA were prepared to the final concentrations of 0, 0.025, 0.05, 0.1, 0.2 and 0.4 mg / mL. The standards were diluted in 0.5% Tween in PBS buffer, incubated in a 70 ºC water bath for 5 min and cooled on ice for 2 min. For blank absorbance readings, 3 technical replicates with 25 µL of sample and standard dilutions were transferred into 96 well plates and absorbance was measured at 562 nm using the Synergy HT spectrophotometer from BioTek Instruments. For measurements, 200

µL of BCA reagent mix at room temperature was added to each well and the plates were shaken for 30 sec and incubated for 30 min in the spectrophotometer at 37 ºC. At the end of the incubation period, absorbance was measured at 562 nm. The software used for procedure setup and data acquisition was Gen5 1.10 from BioTek.

The blank absorbance values of each replicate were subtracted from the final absorbance value and the mean value for the 3 technical replicates of each sample was calculated. A standard curve was traced plotting the absorbance values of the 6 standard concentrations against their respective concentrations. The standard concentration with the absorbance value closest to the sample absorbance values was used to calculate the sample concentrations. Sample concentrations were calculated according to $[sample] = \frac{A(sample)}{A(standard)} * [standard]$.

The total triglyceride and protein values obtained as described above were utilized to estimate strain specific triglyceride content. For each sample, the value of total triglyceride concentration was divided by the value of total protein concentration to obtain the triglyceride-protein ratio that was used for strain/treatment comparisons ($relative\ TAG\ levels = \frac{total[triglyceride]}{total[protein]}$).

## 2.9 – *foraging* RNA and Protein Extraction

To quantify differences in *foraging* RNA and protein in the different strains, total RNA and total protein was extracted from fed and 24 h food deprived *EHMT*$^{+}$*;for*$^{R}$, *EHMT*$^{+}$*;for*$^{s}$, *EHMT*$^{+}$*;for*$^{s2}$, *EHMT*$^{DD1}$*;for*$^{R}$, *EHMT*$^{DD1}$*;for*$^{s}$ and *EHMT*$^{DD1}$*;for*$^{s2}$, *for*$^{R}$, *for*$^{s}$ and *for*$^{s2}$ flies.

The parental generation was reared as in 2.6. Test animals were collected on the first 3 days of eclosion using $CO_2$ anesthesia and aged for 5 - 6 days in nutrient vials with an n = 20 in each vial. Test animals were changed over to fresh nutrient vials every 24 h, and 48 h before the test females in each vial were allowed to mate with 10 males (these males were not used in the test). 5 - 6 days old test females were divided into 2 experimental groups, the first group was transferred fresh nutrient vials and the second group was transferred into food deprivation vials. After 24 h, flies were transferred into 1.5 mL eppendorf tubes in groups of 10 and frozen at -80 ºC.

Total RNA and protein was extracted from the same samples using the TRIzol reagent from Life Technologies (#15596-026). The procedure was modified from the manufacturers' instructions. Samples were removed from -80 ºC and homogenized using a plastic pestle in 50 µL of TRIzol (10 flies weigh approximately 50 mg; which is within the range suggested by the manufacturer) until there were no visible chunks of tissue left. Following homogenization, samples were centrifuged at 4 ºC and 12000 g for 10 min to precipitate particles of cuticle and wings and separate excess fat. The supernatant was removed and transferred into fresh 1.5 mL eppendorf tubes and incubated at room temperature for 5 min. 100 µL of chloroform were added to the homogenate, the tubes were mixed by hand for 15 sec and incubated at room temperature for 2 - 3 min. Samples were centrifuged at 4 ºC

and 12000 g for 15 min to permit the separation of the aqueous (nucleic acids) and the organic phase (proteins). The upper aqueous phase was transferred into new eppendorf tubes and the organic and interphase where kept for protein extraction.

For RNA extraction, 250 µL of isopropanol was added to the aqueous layer and samples were mixed and incubated at room temperature for 10 min. Samples were centrifuged at 4 ºC and 12000 g for 10 min and the supernatant was removed. The pellet was vortexed in 500 µL of 75 % ethanol and re-centrifuged at 4 ºC and 7500 g for 5 min. The ethanol was removed and the pellet was air dried. The precipitated RNA was dissolved in 20 µL of RNAse free water by pipetting and incubating at 55 - 60 ºC for 10 min. RNA was quantified using the Nanodrop 2000c from Thermo Scientific and stored at -80 ºC.

For protein extraction 150 µL of ethanol was added to the non-aqueous portion (organic and interphase), and tubes were mixed by inversion. Samples were incubated at room temperature for 2 - 3 min and centrifuged at 4 ºC and 20000 g for 5 min to remove DNA from the solution. The supernatant was transferred into new eppendorf tubes and 1 mL of isopropanol was added. Samples were stored at room temperature for 10 min and centrifuged at 4 ºC and 12000 g for 10 min. The supernatant was removed and the pellet was washed 3 times with 1 mL of 0.3 M guanidine hydrochloride in 95 % ethanol. During each wash cycle the pellet was stored in solution for 20 min at room temperature and then centrifuged at 4 ºC and 7500 g for 5 min. After the last wash cycle the wash solution was removed and samples were stored in 1 mL of ethanol at room temperature for 20 min. Samples were centrifuged at 4 ºC and 7500 g for 5 min and left to air dry for 5-10 min. The protein pellet was dissolved in 1 mL of 0.1 % SDS by pipetting and incubating at 50 ºC for 10 min. The Supernatant with the soluble proteins was transferred into new eppendorf tubes and quantified using the BCA kit and instructions described in 2.8. Proteins were stored at -20 ºC until further use.

## 2.10 – *foraging* RNA Quantification

Quantitative Real-Time Polymerase Chain (qRT-PCR) was performed utilizing the CFX384 Real-Time PCR Detection System from Bio-Rad (cat#185-5384). For this, total RNA samples were treated with RQ1 RNase-Free DNase from Promega (cat#M6101) to avoid genomic DNA contamination. 1 µL of DNase was added to each sample and samples were incubated at 37 ºC for 30 min, the reaction was terminated by heating the samples to 65 ºC for 10 min. A volume equivalent to 2 µg of total RNA was used for cDNA synthesis using the SuperScriptTM III Reverse Transcriptase from Invitrogen (cat#18080-044) and following manufacturers' instructions. Briefly, 20 µL reactions were set up in 0.2 mL nuclease-free micro centrifuge tubes using 1 µL of 50 µM oligo$(dT)_{20}$ primers, 2 µg total RNA, 1 µL of 10 mM dNTP Mix and 14 µL of nuclease free water. The mixture was heated to 65 °C for 5 min and incubated on ice 1 min. The contents of the tubes were collected by brief centrifugation and 4 µL of 5X First-Strand Buffer, 1 µL of 0.1 M DTT and 1 µL of reverse transcriptase were added to the reaction. The samples were mixed by pipetting and incubated at 50 °C for 60 min. Finally, the reaction enzymes were inactivated the by heating at 70 °C for 15 min.

The Bio-Rad IQ SYBR Green Supermix (Cat# 170-8880S) was used for qRT-PCR with gene specific primers, as shown in Table 2.1. Three pairs of *foraging* specific primers were designed using annotated gene model from FlyBase Release R5.32 Sep 2010 with Primer 3 in Geneious (Drummond *et al,* 2010). Primer pairs specific for *actin5c, rp49* and *α-tubulin* genes were utilized as reference. 10 µL reactions were set up in 384 well plates as follows: a master mix was made out of 5 µL of SYBR Green, 0.2 µL of each the forward and reverse primer (0.2 pM) and 1.8 µL of nuclease free water per reaction. The cDNA was diluted 1:9 in nuclease free water and 1 µL of cDNA was added to each reaction. All PCR experiments were made under the same conditions and with at least three replicates per genotype/primer combination including negative controls ($H_2O$ as template) for all primer combinations. The cycling protocol included an initial denaturation at 95 ºC for 3 min, 45 cycles; denaturing at 95 ºC for 3 sec and annealing-extension at 60 ºC for 30 sec, and a final melting curve starting at 65 ºC and increasing at 0.5 ºC increments at each step to 95 ºC. All comparisons of gene expression levels were performed on identical cDNA dilutions, using *actin5c, rp49* and *α-tubulin* gene expression as controls. Melt curves were observed to ensure correct amplification products (single dissociation curves) and *foraging* threshold cycles ($C_t$) equilibrated with mean reference gene $C_t$ to calculate $\Delta C_t$ ($\Delta C_t$ = $C_t$ of interest - mean reference gene $C_t$). *foraging* expression levels were analyzed by calculating $\Delta\Delta C_t$ ($\Delta\Delta C_t$= $\Delta C_{ta}$ _mean $\Delta C_{tb}$, where a and b are being compared), which in turn was used to determine mean fold change ($2\text{-}\Delta\Delta C_t$) ± standard deviation between strains, and/or treatments.

**Table 2.1- Oligonucleotides Used for PCR Amplification of *for* Coding Sequences and Reference Genes**

| Gene | Primer designation and Orientation | Primer Sequence | Amplicon Tm |
|---|---|---|---|
| ***foraging*** | q3short - Forward | 5'- TTGATGACTATCCTCCCGATCCT -3' | 82 °C |
| | q3short - Reverse | 5'- CTGACTGTTGCTGTTGGCTTTG -3' | |
| | q3long - Forward | 5'- CACAATCATCCATCGTCGTAGC -3' | 76 °C |
| | q3long - Reverse | 5'- TACAATGCACATGGCTAACAGAGC -3' | |
| | qPcom - Forward | 5'- GGACACGCAGGAGGAGAAGT -3' | 83 °C |
| | qPcom - Reverse | 5'- GTCGTAGCGATGCTTGATCTC -3' | |
| ***actin 5C*** | Actin5c - Forward | 5'- TGAGCGTGAAATCGTCCGTGA -3' | 86 °C |
| | Actin5c - Reverse | 5'- CGCAAGCCTCCATTCCCAAGA -3' | |
| ***alpha tubulin 84B*** | atub - Forward | 5'- GCAGGGCTTCCTCATCTTCCA -3' | 87 °C |
| | atub - Reverse | 5'- CAGGGTGGTGTGGGTGGTCA -3' | |
| **ribosomal protein 49** | rp49 - Forward | 5'- ATCGGTTACGGATCGAACAA -3' | 85 °C |
| | rp49 - Reverse | 5'- GACAATCTCCTTGCGCTTCT -3' | |

## 2.11 – *foraging* Protein Quantification

The protein extractions from strains $EHMT^{+};for^{R}$, $EHMT^{+};for^{S}$, $EHMT^{DD1};for^{R}$ and $EHMT^{DD1};for^{S}$ obtained in 2.9 were analyzed by western blotting to determine possible differences in FOR protein concentration. 10 µg of total protein were separated by denaturing polyacrylamide gel electrophoresis and transferred onto BioTrace NT pure nitrocellulose (cat# 66485) using the Bio-Rad Mini Trans-Blot system (cat# 170-3930), following the manufacturer's instructions. Immunodetection of FOR protein was carried out as in (Belay *et al*, 2007) using polyclonal rabbit anti-FOR and horseradish peroxidase-conjugated goat anti-rabbit or anti-guinea pig IgG (Jackson Immunological, West Grove, PA) at 1:1,000 and 1:10,000 (v/v) dilutions, respectively. The lower portions of the membranes where the bands corresponding to the approximated size of Actin were separated from the rest and immunodetection of Actin was carried out using mouse (mab) anti-Actin and horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immunological, West Grove, PA) ) at 1:1,000 and 1:10,000 (v/v) dilutions, respectively. Signals were detected using the ECL Plus Western Blotting Detection System, following manufacturers' instructions.

## 2.12 – Statistical Analysis

Statistical program Sigmaplot 11.0 was utilized for all statistical analysis. Differences in means between test groups and/or treatments were assessed by Analysis of variance (ANOVA). In cases where data failed to pass the Shapiro-Wilk normality test (non-parametric data), either Kruskal-Wallis one way analysis of variance on ranks with pairwise multiple comparison procedures following the Dunn's Method, or two and three way ANOVAs with pairwise multiple comparison procedures following the Holm-Sidak and the Student-Newman-Keuls methods were used.

# 3 – Results

## 3.1 – Developmental effects of *EHMT$^{DD1}$* and *for$^{R}$*

Crossing schemes allowed for successful generation of homozygous *EHMT$^{+}$;for$^{R}$*, *EHMT$^{+}$;for$^{S}$*, *EHMT$^{+}$;for$^{S2}$*, *EHMT$^{DD1}$;for$^{S}$* and *EHMT$^{DD1}$;for$^{S2}$* lines. However, after crossing out the *CyO* balancer chromosome, the self-crossing of homozygous *EHMT$^{DD1}$;for$^{R}$* males and females did not result in viable populations. To further investigate possible synergistic effects of *EHMT* mutants and *for*, the viability of all strains was compared at various developmental stages, from egg to adult.

For this purpose, 100 eggs were collected for each strain and 5 assay repetitions were analyzed. The mean percentage ± standard deviation of eggs hatched are shown in Table 3.1, and were 79 ± 5.7 for *for$^{R}$*, 60 ± 10.2 for *for$^{S}$*, 51 ± 7.1 for *for$^{S2}$*, 30 ± 6.8 for *EHMT$^{+}$;for$^{R}$*, 31 ± 5.8 for *EHMT$^{+}$;for$^{S}$*, 25 ± 8.1 for *EHMT$^{+}$;for$^{S2}$*, 40 ± 4.2 for *EHMT$^{DD1}$;for$^{S}$* and 15 ± 11.5 for *EHMT$^{DD1}$;for$^{S2}$*. Although homozygous *EHMT$^{DD1}$;for$^{R}$* laid roughly the same number of eggs, 0 % of these eggs hatched and consequently this strain gave no progeny. Statistical analysis on the hatchability showed significant differences between the laboratory control lines (one-way ANOVA: $F_{(2)} = 16.396$, $p < 0.001$) and post-hoc tests showed that rovers had significantly higher hatchability than sitters (SNK: $q = 5.382$, $p = 0.003$) and *for$^{S2}$* (SNK: $q = 7.931$, $p < 0.001$), while sitters and *for$^{S2}$* did not significantly differ from each other (SNK: $q = 2.549$, $p = 0.097$). The ANOVA on the hatchability scores of *EHMT* mutants and revertants showed that there was a significant difference between these strains (one-way ANOVA: $F_{(5)} = 20.262$, $p < 0.001$) and post-hoc analysis revealed that this was due to differences in the *EHMT* mutants. The differences between rover, sitter and *for$^{S2}$ EHMT* revertants were not significant (SNK: $q = 0.319$, $p = 0.824$ for *EHMT$^{+}$;for$^{R}$* vs *EHMT$^{+}$;for$^{S}$* ; $q = 1.593$, $p = 0.271$ for *EHMT$^{+}$;for$^{R}$* vs *EHMT$^{+}$;for$^{S2}$* and $q = 1.912$, $p = 0.381$ *EHMT$^{+}$;for$^{S}$* vs *EHMT$^{+}$;for$^{S2}$*) but *EHMT* mutants were all significantly different from each other (SNK: $q = 12.748$, $p < 0.001$ for *EHMT$^{DD1}$;for$^{R}$* vs *EHMT$^{DD1}$;for$^{S}$* ; $q = 4.780$, $p = 0.003$ for *EHMT$^{DD1}$;for$^{R}$* vs *EHMT$^{DD1}$;for$^{S2}$* and $q = 7.967$, $p < 0.001$ *EHMT$^{DD1}$;for$^{S}$* vs *EHMT$^{DD1}$;for$^{S2}$*) with sitter showing the highest hatchability. Interestingly, the loss of *EHMT* only significantly affected hatchability scores in the rover 2$^{nd}$ chromosome background (SNK: $q = 9.561$, $p < 0.001$), while strains with the sitter (SNK: $q = 2.868$, $p = 0.054$) and *for$^{S2}$* (SNK: $q = 3.187$,

p = 0.054) 2nd chromosome backgrounds were not significantly affected. The hatchability problems observed in *EHMT$^{DD1}$;for$^{R}$* do not occur when the 2nd chromosome is balanced with the *CyO* balancer chromosome, evident as these flies give rise to healthy populations with a ratio of 1:3 homozygous : heterozygous flies. This confirms that it is only when flies are homozygous for the rover 2nd chromosome, that the lack of *EHMT* generates fertility problems, even though homozygous *EHMT$^{DD1}$;for$^{R}$* originating from heterozygous parents exhibit seemingly normal development . For all other strains the survival until pupation and to adulthood of the hatched larva was close to 100 %. This suggests synergistic effects of *EHMT* and *for* on fertility, specific to the rover allele and this interaction affects egg-hatchability and not survivorship to pupation or adulthood.

**Table 3.1 – Percentage viability at various developmental stages**

| Strain | Mean % ± STDV of hatched eggs | Mean % ± STDV of survival until pupation | Mean % ± STDV survival until adulthood |
|---|---|---|---|
| *for$^{R}$* | 79 ± 5.7 | 99 ± 0.5 | 99 ± 0.4 |
| *for$^{S}$* | 60 ± 10.2 | 99 ± 0.8 | 98 ± 0.6 |
| *for$^{S2}$* | 51 ± 7.1 | 97 ± 0.7 | 96 ± 0.9 |
| *EHMT$^{+}$;for$^{R}$* | 30 ± 6.8 | 93 ± 1.2 | 92 ± 1.1 |
| *EHMT$^{+}$;for$^{S}$* | 31 ± 5.8 | 99 ± 0.6 | 98 ± 0.5 |
| *EHMT$^{+}$;for$^{S2}$* | 25 ± 8.1 | 98 ± 0.5 | 98 ± 0.5 |
| *EHMT$^{DD1}$;for$^{R}$* | 0 ± 0 | - | - |
| *EHMT$^{DD1}$;for$^{S}$* | 40 ± 4.2 | 93 ± 0.8 | 91 ± 0.9 |
| *EHMT$^{DD1}$;for$^{S2}$* | 15± 11.5 | 95 ± 1.1 | 91 ± 0.8 |

## 3.2 – Homozygous *EHMT$^{DD1}$;for$^{R}$* males and females are sterile

To further analyze the sterility *EHMT$^{DD1}$;for$^{R}$* strain, homozygous *EHMT$^{DD1}$;for$^{R}$* were crossed to wild type rover flies. In all 5 test runs, no living larvae hatched from the laid eggs, the mean of hatched larvae being effectively 0 ± 0 larva. This was the case for both homozygous *EHMT$^{DD1}$;for$^{R}$* females crossed with wild type *for$^{R}$* males and homozygous *EHMT$^{DD1}$;for$^{R}$* males crossed with wild type *for$^{R}$* females. Since progeny originating from these crosses would have one functional allele of the *EHMT* (i.e. express *EHMT*), and therefore be viable, it is unlikely that this effect is due to embryonic lethality. This means that homozygous *EHMT$^{DD1}$;for$^{R}$* males and females (i.e. Flies without a functional *EHMT* allele and a *for$^{R}$* second chromosome) are sterile.

## 3.3 – The loss of functional *EHMT* affects larval foraging behaviour

In the first of the two larval path length tests, significant differences were found in mean larval path lengths between strains and food treatments (fed and food deprived). One-way-ANOVAs established no significant effects of sex (one-way ANOVA on ranks; $H_{(1)}$ = 0,000701; p = 0.979) and day of testing (two-way ANOVA on ranks; $F_{(3,2)}$ = 1.675; P = 0.124) on larval path length for each strain, allowing the data to be pooled. Data showing pooled data of mean path lengths ± standard error are shown in Figure 3.3.1. These values were 9.13 ± 0.21 for $for^R$ fed, 4.22 ± 0.25 for $for^R$ food-deprived, 4.64 ± 0.22 for $for^S$ fed, 1.12 ± 0.10 for $for^S$ food deprived, 4.56 ± 0.18 for $EHMT^+$ fed, 1.36 ± 0.13 for $EHMT^+$ food deprived, 4.97 ± 0.18 for $EHMT^{DD1}$ fed and 1.77 ± 0.13 for $EHMT^{DD1}$ food-deprived. The ANOVA on ranks (see S1 for values) showed that there was a significant effect of food-deprivation on path length for all strains. As expected, rovers have significantly longer path lengths than sitters both in fed and food deprived conditions, and there were no significant differences between food-deprived rovers and fed sitters. Interestingly, there were no significant differences between $EHMT^+$ and $EHMT^{DD\ 1}$ strains in fed or food-deprived condition, and both strains had significantly lower path lengths than the control rovers and were not significantly different from the control sitters.

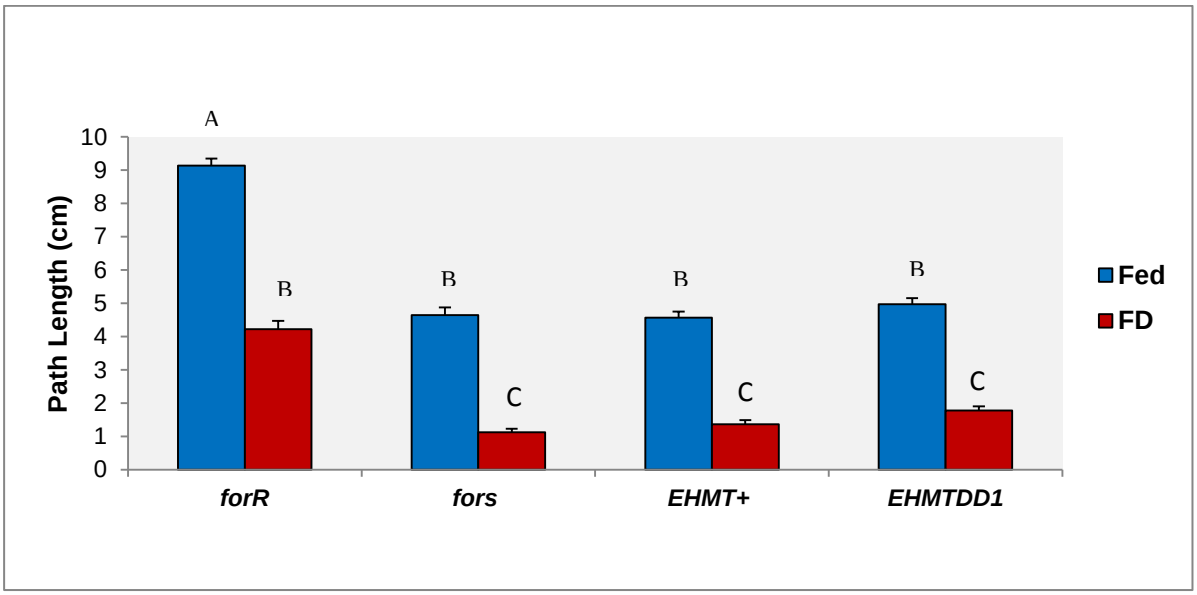


**Figure 3.3.1 – Larval foraging experiment 1. Mean larval path length (centimeters) ± SE for strains $for^R$, $for^S$, $EHMT^+$ and $EHMT^{DD1}$. Pooled data for sex and for 3 replicate days where 110 < n < 120 larva were tested per strain. Letters represent statistical groups and means with the same letter are not significantly different. Fed and FD stand for fed and food deprived conditions.**

In the second larval path length experiment, the *EHMT* mutant and revertant strains with controlled 2nd chromosome backgrounds (homozygous for the $for^R$, $for^s$ or $for^{s2}$ alleles) were tested. In this experiment, the results for the standard laboratory control lines $for^R$, $for^s$ and $for^{s2}$ (figure 3.3.2) were as expected. The data was tested for day effects over the 5 replicate test days, and there was a slight day effect for $for^{s2}$ on day 5, so the data from that day was omitted in further analysis. The mean larval path length, plus or minus standard error, was 10.14 ± 0.23 for $for^R$, 7.10 ± 0.21 for $for^s$ and 7.11 ± 0.23 for $for^{s2}$. These values show that sitters and $for^{s2}$ had significant lower path lengths while foraging than rovers (comparisons of means by Holm-Sidak method: DM = 3.012; t = 9.723; $p < 0.001$ for sitters and DM = 3.273; t = 9.712; $P<0.001$ for $for^{s2}$). Sitters and $for^{s2}$ were not significantly different from each other (comparisons of means by Holm-Sidak method: DM = 0.260; t = 0.777; p = 0.982).

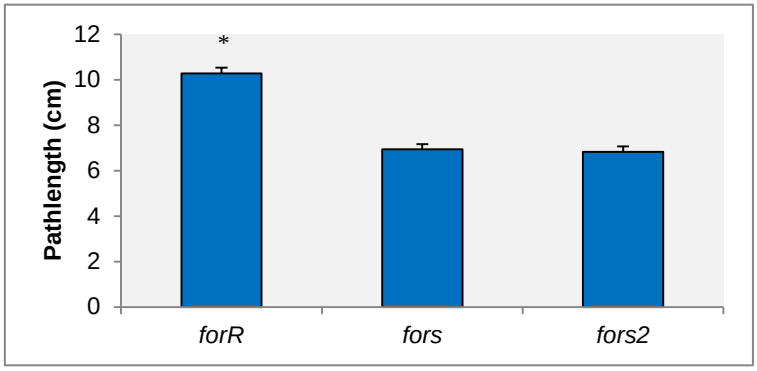


**Figure 3.3.2 – Controls for second larval foraging experiment. Mean larval path length (centimeters) ± SE for strains $for^R$, $for^s$, and $for^{s2}$. Data pooled over 4 replicate days and 130 < n < 140 larva/strain. Bars with asterisks show significant differences in means.**

Figure 3.3.3 illustrates the complete results for second larval foraging experiment, showing the comparison between fed and food-deprived larvae of all strains (Figure 3.3.3 a) as well as comparisons between *EHMT* mutants and revertants in fed (Figure 3.3.3 b) and food-deprived (Figure 3.3.3 c) conditions. The 4 h food-deprivation prior to the test resulted in significant lower path lengths in all strains (two-way ANOVA: $F_{(5,1)} = 11.819$, $p < 0.001$; for all pairwise comparisons between strains and treatments see S2). Interestingly, even though there were no significant differences in mean path length between *EHMT* mutant and revertants in the first experiment where the second chromosomes were not substituted into the genetic background, statistical analysis showed significant differences between the *EHMT* revertants ($EHMT^+;for^R$, $EHMT^+;for^s$ and $EHMT^+;for^{s2}$) and the *EHMT* mutants ($EHMT^{DD1};for^R$, $EHMT^{DD1};for^s$ and $EHMT^{DD1};for^{s2}$), regardless of the *for* allele. This was evident as mean larval path lengths, plus or minus standard errors of 9.96 ± 0.24 for $EHMT^+;for^R$, 6.83 ± 0.25 for

*EHMT*$^{+}$*;for*$^{s}$, 5.92 ± 0.24 for *EHMT*$^{+}$*;for*$^{s2}$, 9.89 ± 0.32 for *EHMT*$^{DD1}$*;for*$^{R}$, 7.90 ± 0.21 for *EHMT*$^{DD1}$*;for* $^{s}$, and 5.84 ± 0.27 for *EHMT*$^{DD1}$*;for*$^{s2}$ in the fed condition and 5.97 ± 0.24 for *EHMT*$^{+}$*;for*$^{R}$, 2.69 ± 0.24 for *EHMT*$^{+}$*;for*$^{s}$, 2.42 ± 0.25 for *EHMT*$^{+}$*;for*$^{s2}$, 7.61 ± 0.33 for *EHMT*$^{DD1}$*;for*$^{R}$, 5.81 ± 0.26 for *EHMT*$^{DD1}$*;for* $^{s}$, and 4.79 ± 0.26 for *EHMT*$^{DD1}$*;for*$^{s2}$ in the food-deprived condition.

The rover-sitter differences observed in the *EHMT* mutant and revertant lines followed the same patterns as the laboratory control lines. Rovers had significantly longer path lengths than sitters in the *EHMT* revertant background as well as in the mutant background. This was the case for both fed larvae (comparisons of means by Holm-Sidak method: DM = 3.135; t = 9.135; $p < 0.001$ for *EHMT* revertants and DM = 1.989; t = 5.171; $p < 0.001$ for *EHMT* mutants) and food-deprived larvae (comparisons of means by Holm-Sidak method: DM = 3.274; t = 9.743; $p < 0.001$ for *EHMT* revertants and DM = 1.798; t = 4.266; $p < 0.001$ for *EHMT* mutants).

Results of larval path-length tests for fed larvae are shown in Figure 3.3.3 b. ANOVAs showed that *EHMT* mutant larvae were not significantly different from the revertants on rover and *for*$^{s2}$ second chromosome backgrounds (comparisons of means by Holm-Sidak method: DM = 0.0714; t = 0.178; $p = 0.859$ for rovers and DM = 0.0793; t = 0.221; $p = 0.970$). However, there was a significant increase in path length while foraging caused by the loss of *EHMT* on a sitter 2$^{nd}$ chromosome background (comparisons of means by Holm-Sidak method: DM = 1.074; t = 3.336; $p = 0.004$). In food-deprived larvae (Figure 3.3.3 c), the loss of *EHMT* resulted in significantly longer path lengths independent of the second chromosome background (comparisons of means by Holm-Sidak method: DM = 1.639; t = 4.002; $p < 0.001$ for rover; DM = 3.114; t = 8.884; $p < 0.001$ for sitter and DM = 2.368; t = 6.634; $p < 0.001$ for *for*$^{s2}$).

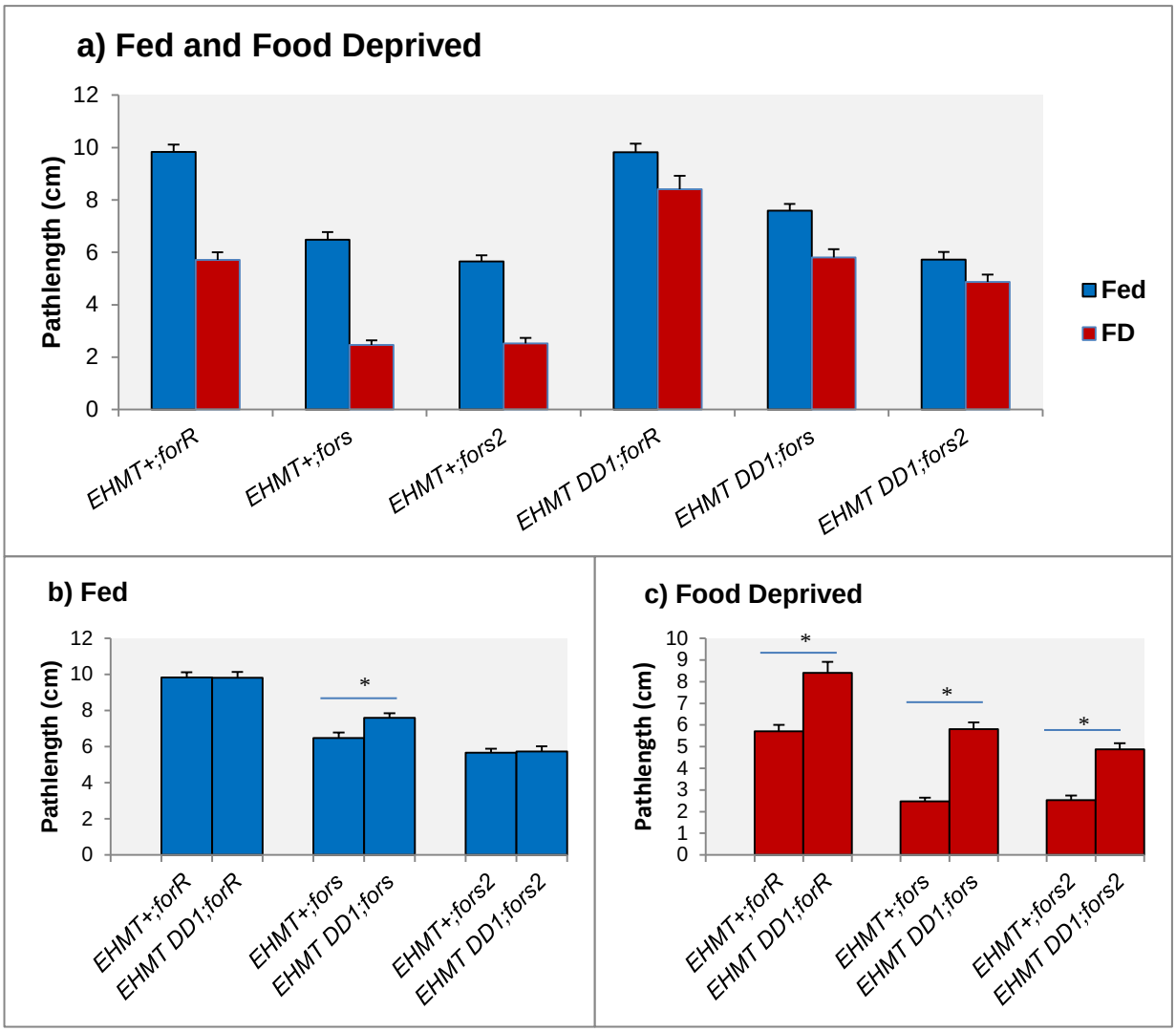


**Figure 3.3.3 - Mean larval path length (centimeters) ± SE for strains *EHMT$^{+}$;for$^{R}$*, *EHMT$^{+}$;for$^{S}$*, *EHMT$^{+}$;for$^{S2}$*, *EHMT$^{DD1}$;for$^{R}$*, *EHMT$^{DD1}$;for$^{S}$* and *EHMT$^{DD1}$;for$^{S2}$*, demonstrating: a) Comparison between fed and food deprived larvae b) Comparison between strains in fed larvae c) Comparison between strains in 4h food deprived larvae. Data pooled over 5 replicate days for all strains. 130 < n < 140 larva/strain. Bars with asterisks show significant differences in means**

## 3.4 – The loss of *EHMT* affects adult foraging behaviour

The adult foraging behaviour results of control laboratory strains as well as *EHMT* mutant and revertant flies with the three 2nd chromosome backgrounds ($for^R$, $for^s$ and $for^{s2}$) are shown in Figure 3.4. The mean number ± SE of found and consumed sucrose drops by 24 h food-deprived adult females over a 5 and 10 minute test period were 1.63 ± 0.15 for $for^R$, 0.37 ± 0.11 for $for^s$, 0.53 ± 0.14 for $for^{s2}$, 1.33 ± 0.13 for $EHMT^+;for^R$, 0.63 ± 0.12 for $EHMT^+;for^s$ , 1.3 ± 0.23 for $EHMT^+;for^{s2}$, 1.67 ± 0.12 for $EHMT^{DD1};for^R$, 1.73 ± 0.19 for $EHMT^{DD1};for^s$ and 0.8 ± 0.18 for $EHMT^{DD1};for^{s2}$ at 5 min and 2.33 ± 0.16 for $for^R$, 0.7 ± 0.12 for $for^s$, 1.13 ± 0.19 for $for^{s2}$, 2.1 ± 0.14 for $EHMT^+;for^R$, 1.17 ± 0.14 for $EHMT^+;for^s$,1.9 ± 0.24 for $EHMT^+;for^{s2}$, 2.37 ± 0.18 for $EHMT^{DD1};for^R$, 2.27 ± 0.2 for $EHMT^{DD1};for^s$ and 1.27 ± 0.21 for $EHMT^{DD1};for^{s2}$ at 10 min. Since the data was non-parametric, Kruskal-Wallis One Way Analysis of Variance on Ranks was used to test for strain differences at 5 and 10 min. There was a statistically significant difference among the median values of the different strains (one-way ANOVA on ranks: $H_{(8)} = 79.186$; $p < 0.001$), so the Newman-Keuls Method (SNK) was used for post-hoc analysis to indicate which strains differed from each other.

As shown in Figure 3.4 a), after 5 minutes testing the laboratory wild type rovers found and consumed significantly more sucrose drops than sitters (SNK: $q = 9.092$; $p < 0.05$). The $for^{s2}$ strain behaved as expected, being significantly different from rover (SNK: $q = 9.163$; $p < 0.05$) and not significantly different from sitters (SNK: $q = 4.297$; $p > 0.05$). After 10 min testing time, rovers still consumed significantly more sucrose drops than sitters (SNK: $q = 8.049$; $p < 0.05$), and $for^{s2}$ had significantly lower scores than rovers (SNK: $q = 6.643$; $p < 0.05$) and significantly higher scores than sitters (SNK: $q = 9.581$; $p < 0.05$).

The values for the *EHMT* revertant and mutant strains (figure 3.4 b) showed that $EHMT^+;for^R$, $EHMT^+;for^s$ behave the same way as the rover and sitter wild types, with the *EHMT* revertant on a rover background finding and consuming significantly more sucrose drops than the revertant on a sitter background (SNK: $q = 9.169$; $p < 0.05$ at 5 min and $q = 10.702$; $p < 0.05$ at 10 min). Contrary to what would be expected considering the wild type data, the *EHMT* revertant on the $for^{s2}$ background behaved like the revertant on the rover background, evident as $EHMT^+;for^{s2}$ and $EHMT^+;for^R$ not being significantly different from each other (SNK: $q = 4.616$; $p > 0.05$ at 5 min and $q = 4.501$; $p > 0.05$ at 10 min) while $EHMT^+;for^{s2}$ and $EHMT^+;for^s$ were significantly different at both 5 minutes (SNK: $q = 9.124$; $p < 0.05$) and 10 minutes (SNK: $q = 11.241$; $p < 0.05$).

Interestingly, the loss of *EHMT* in the *EHMT* mutants with the rover 2nd chromosome had no significant effects (SNK: $q = 3.648$; $p > 0.05$ at 5 min and $q = 2.977$; $p > 0.05$ at 10 min) but in flies with the sitter 2nd chromosome background the *EHMT* mutants consumed significantly more drops than the revertants (SNK: $q = 8.229$; $p < 0.05$ at 5 min and $q = 9.376$; $p < 0.05$ at 10 min). This increase in sitter foraging activity resulted in the loss of rover-sitter differences in the *EHMT* null mutants (i.e. $EHMT^{DD1};for^R$ and $EHMT^{DD1};for^s$ were not different with SNK: $q = 0.977$; $p > 0.05$ at 5 min and $q = 2.446$; $p > 0.05$ at 10 min). Unexpectedly, the absence of *EHMT* in the $EHMT^{DD1};for^{s2}$ mutants caused a decrease in the number of sucrose drops consumed (SNK: $q = 14.327$; $p < 0.05$ at 5 min and $q = 10.763$; $p < 0.05$ at 10 min.

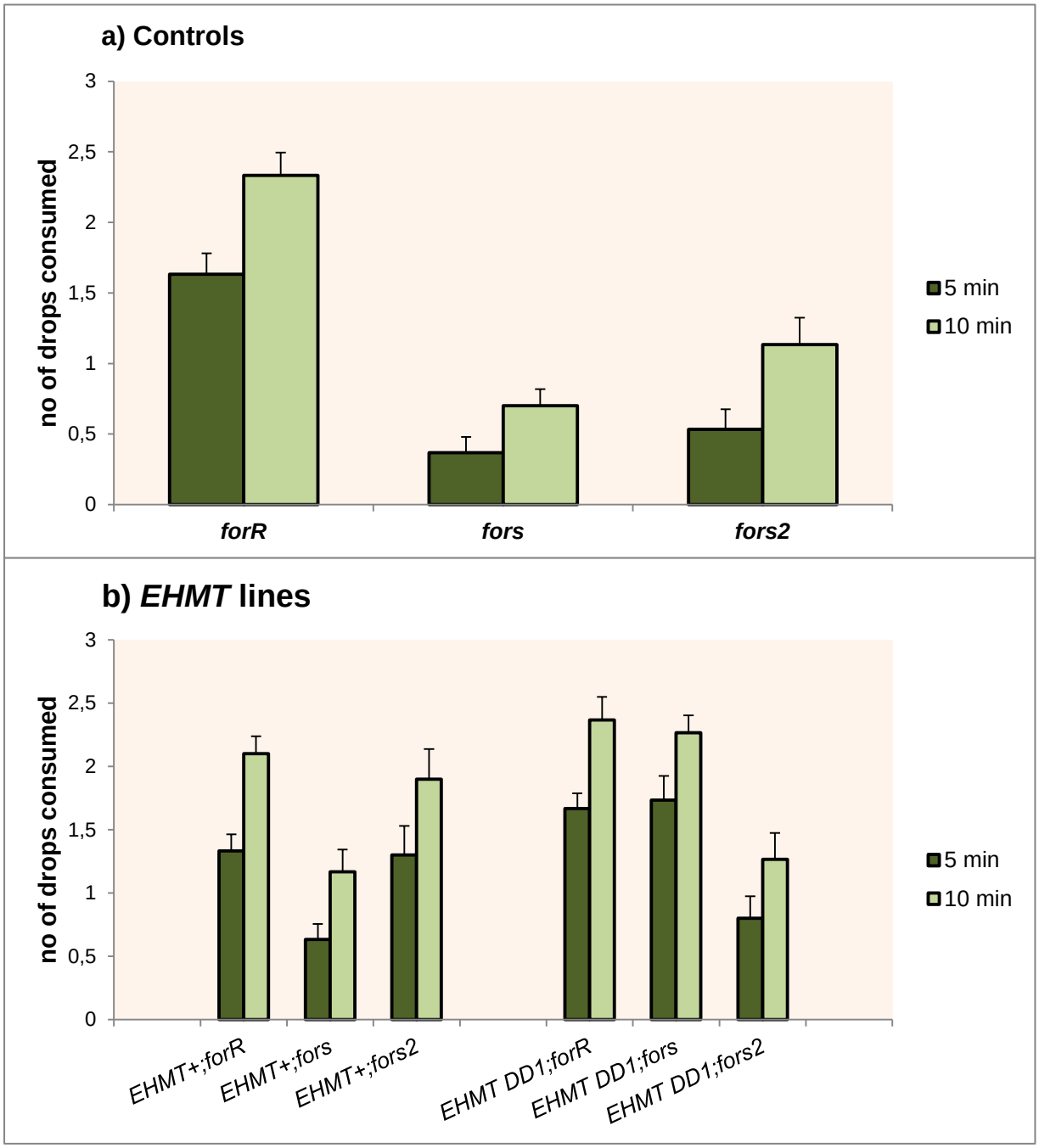


**Figure 3.4 – Mean sucrose drops consumed ± SE for all strains, demonstrating a) Laboratory rover, sitter and *for*$^{s2}$ controls at 5min and 10min. b) *EHMT* mutants and revertants on rover, sitter and *for*$^{s2}$ backgrounds at 5 min and 10 min. Data collected over 10 days with 24h food deprived 5-6 days old females. n = 30 individuals/strain. Bars with asterisks show significant differences in means.**

## 3.5 - *EHMT* mutants survive longer under food deprivation

Starvation resistance assays over a 150 h starvation period were executed and analyzed for males and females separately since females exhibit a much higher resistance to starvation than males due to fundamental differences in body size and fat storage. Because of possible genetic background effects on starvation, comparisons were only made within the *EHMT* mutant and revertant lines and the laboratory control lines were used as an internal control for rover-sitter phenotypes.

Results for females of all strains are illustrated in figure 3.5.1, where a) shows the laboratory control females and b) shows the *EHMT* revertant and mutant females. Inserts in the right top corner show time points to 80, 50 and 20 % alive. The results indicated that sitter females survived longer than rovers, although the rate of death was not different after the first fly died, evident as survival curves with similar slopes with the sitter curve being shifted to later time points. However, in $for^{s2}$ females the period between the death of the first and the last flies was longer than that of rovers and sitters, resulting in a less sloped survival curve.

To further analyze differences in survival rates between strains, 3 time points (time to 80, 50 and 20 % alive) were chosen for statistical analysis. ANOVAs showed significant differences between the control rover, sitter and $for^{s2}$ strains at all 3 time points tested: time to 80% alive (one-way ANOVA: $F_{(2)} = 30.265$; $p < 0.001$), time to 50% alive (one-way ANOVA on ranks: $H_{(2)} = 24.106$; $p < 0.001$) and time to 20% alive (one-way ANOVA on ranks: $H_{(2)} = 24.475$; $p < 0.001$). Post-hoc analysis showed that sitter females survived longer than rovers, with significant differences at all 3 time points (comparisons of means by Holm-Sidak method: DM = 28.80; t = 6.453; $p < 0.001$ for 80% alive, SNK: q = 6.789; $p < 0.05$ for 50% alive and q = 6.879; $p < 0.05$ for 20 % alive). $for^{s2}$ females showed significant difference in the time to 80% alive to sitters (comparisons of means by Holm-Sidak method: DM = 31.20; t = 6.991; $p < 0.001$) but not to rovers (comparisons of means by Holm-Sidak method: DM = 2.40; t = 0.538; $p = 0.595$). At the rest of the time points $for^{s2}$ females showed significant higher survivorship than rovers (SNK: q = 4.169; $p < 0.05$ for 50% alive and q = 4.677; $p < 0.05$ for 20 % alive) and significant lower survivorship than sitters (SNK: q = 5.933; $p < 0.05$ for 50% alive and q = 5.559; $p < 0.05$ for 20 % alive).

The *EHMT* revertant and mutant females with $for^R$ and $for^s$ alleles displayed the same rover-sitter pattern as the control laboratory strains, with similar survival rates and sitters surviving longer than rovers. As statistical analysis showed, the differences between strains were significant (one-way ANOVA on ranks: $H_{(5)} = 32.941$; $p < 0.001$ for time to 80% alive, $H_{(5)} = 48.444$; $p < 0.001$ for time to 50% alive and $H_{(5)} = 47.681$; $p < 0.001$ for time to 20% alive), with rovers dying earlier than sitters in both the *EHMT* mutants (SNK: q = 7.831; $p < 0.05$ for 80% alive, q = 7.617; $p < 0.05$ for 50% alive and q = 7.136; $p < 0.05$ for 20 % alive) and revertants (SNK: q = 4.757; $p < 0.05$ for 80% alive, q = 4.383; $p < 0.05$ for 50% alive and q = 6.468; $p < 0.05$ for 20 % alive). Accordingly, $EHMT^{+};for^{R}$ reached all 3 time points significantly sooner than $EHMT^{+};for^{s}$ and the same was true for $EHMT^{DD1};for^{R}$ and $EHMT^{DD1};for^{s}$. Unexpectedly, $EHMT^{+};for^{s2}$ and $EHMT^{DD1};for^{s2}$ survived significantly less time than the *EHMT* revertants and mutants on rover and sitter backgrounds (for all pairwise comparisons see S3).

Interestingly, as evident from the survival curves, the absence of *EHMT* in the mutant females resulted in significant longer survivorship in comparison to the revertants. This was true for all *foraging* alleles, although the effect seems to be larger in the sitter strains (SNK: $q = 6.938$; $p < 0.05$ for 80% alive, $q = 7.155$; $p < 0.05$ for 50% alive and $q = 6.735$; $p < 0.05$ for 20 % alive) than in the rover strains (SNK: $q = 5.194$; $p < 0.05$ for 80% alive, $q = 5.708$; $p < 0.05$ for 50% alive and $q = 6.397$; $p < 0.05$ for 20 % alive).

Results for males of all strains are illustrated in Figure 3.5.2, where a) shows the laboratory control males and b) shows the *EHMT* revertant and mutant females. Inserts in the right top corner show time points to 80, 50 and 20 % alive. In all cases, assays show that males survived for a much shorter period of time than females. As illustrated in Figure 3.5.2 a), males from the laboratory control strains behaved the same as female flies, with rovers, sitters and $for^{s2}$ displaying similar death rates after the death of the first fly and sitters surviving longer. Statistical analysis showed significant differences between strains at all 3 time points tested (one-way ANOVA on Ranks: $H_{(2)} = 23.409$; $p < 0.001$ for time to 80% alive, $H_{(2)} = 22.209$; $p < 0.001$ for time to 50% alive and one-way ANOVA: $F_{(2)} = 32.327$; $p < 0.001$ for time to 20% alive). Post hoc analysis showed that sitter males survived longer than rover (SNK: $q = 6.681$; $p < 0.05$ for 80% alive, $q = 6.556$; $p < 0.05$ for 50% alive and $q = 11.311$; $p < 0.05$ for 20 % alive) and that $for^{S2}$ males survived significantly longer than rover males (SNK: $q = 4.169$; $p < 0.05$ for 80% alive, $q = 4.998$; $p < 0.05$ for 50% alive and $q = 6.670$; $p < 0.05$ for 20 % alive), and significantly less than sitters (SNK: $q = 5.773$; $p < 0.05$ for 80% alive, $q = 4.757$; $p < 0.05$ for 50% alive and $q = 4.640$; $p < 0.05$ for 20 % alive).

Interestingly, males of the *EHMT* mutant and revertant strains behaved differently than females as demonstrated in Figure 3.5.2 b). Survival curves indicate no differences in survival time or rate between *EHMT* revertants on rover, sitter and $for^{s2}$ 2$^{nd}$ chromosome backgrounds. The *EHMT* mutants survived longer than the revertants on all backgrounds, with sitters displaying a different survival rate after the first fly died than rovers. ANOVAs on the 3 chosen time-points showed that the strains were significantly different from each other (one-way ANOVA on Ranks: $H_{(5)} = 33.809$; $p < 0.001$ for time to 80% alive, $H_{(5)} = 37.412$; $p < 0.001$ for time to 50% alive and one-way ANOVA: $F_{(5)} = 45.381$; $p < 0.001$ for time to 20% alive). Post-hoc multiple comparison procedures showed that there were no significant differences in survivorship of rover, sitter and $for^{s2}$ *EHMT* revertant strains (see S3 for values). In the *EHMT* mutants only $for^{s2}$ differed significantly from rovers and sitters, showing less resistance to starvation than both rovers (SNK: $q = 5.119$; $p < 0.05$ for 80% alive, $q = 5.837$; $p < 0.05$ for 50% alive and $q = 4.167$; $p < 0.05$ for 20 % alive) and sitters (SNK: $q = 1.550$; $p > 0.05$ for 80% alive, $q = 4.543$; $p < 0.05$ for 50% alive and $q = 5.265$; $p < 0.05$ for 20 % alive). Finally, the absence of *EHMT* in the mutant males also resulted in significant longer survivorship in comparison to the revertants in sitters (SNK: $q = 5.410$; $p > 0.05$ for 80% alive, $q = 6.952$; $p < 0.05$ for 50% alive and $q = 7.899$; $p < 0.05$ for 20 % alive) as well as rovers (SNK: $q = 8.480$; $p > 0.05$ for 80% alive, $q = 7.385$; $p < 0.05$ for 50% alive and $q = 8.372$; $p < 0.05$ for 20 % alive).

Taken together, the survival assays showed that the absence of functional *EHMT* increases resistance to starvation in both female and male adult flies.

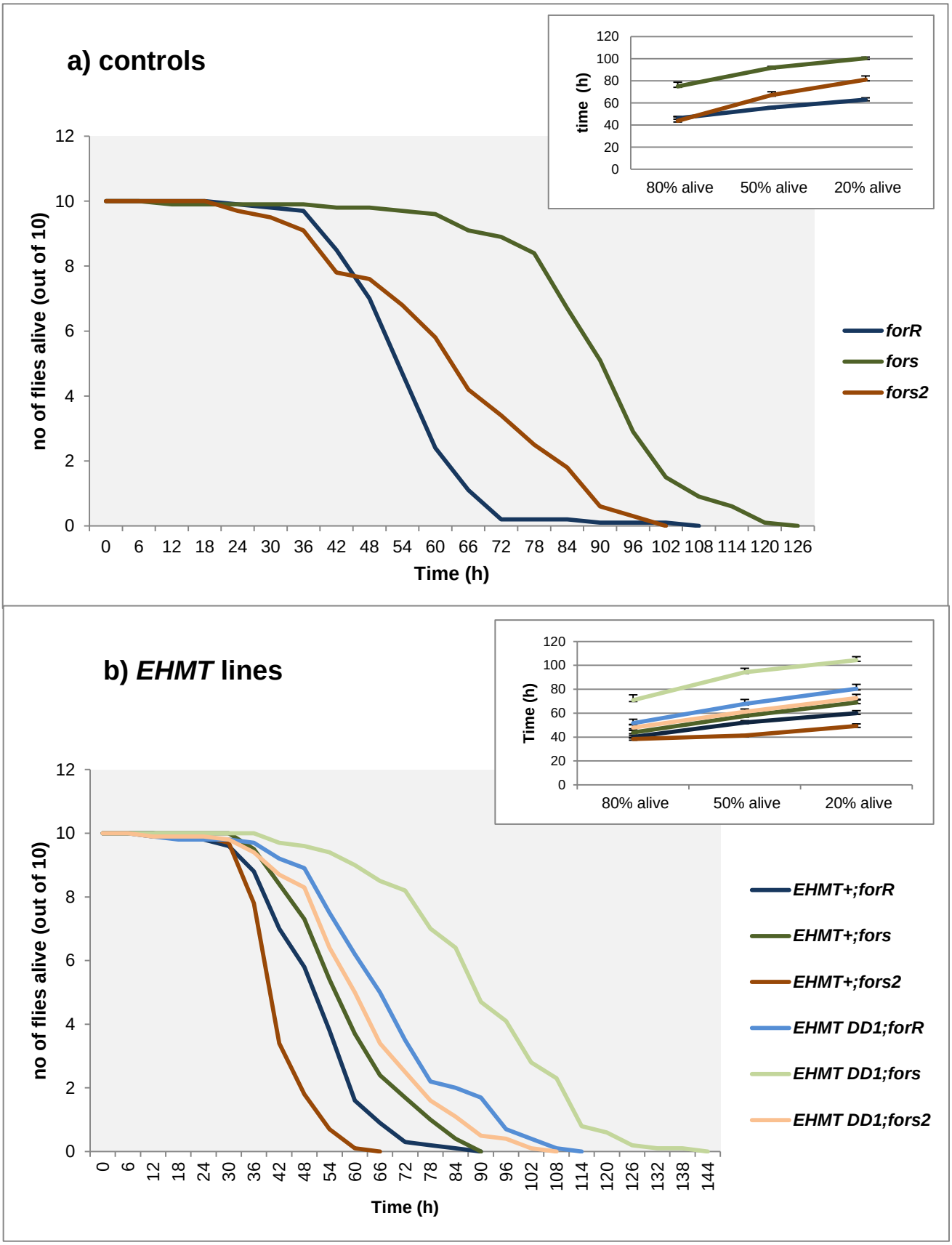


**Figure 3.5.1 – Survival rates for females flies of all strains, demonstrating a) $for^{R}$, $for^{s}$ and $for^{s2}$ control lines and b) $EHMT^{+};for^{R}$, $EHMT^{+};for^{s}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$. Iserts show the mean time (h) to 80%, 50% and 20% of flies alive ± standard error.**

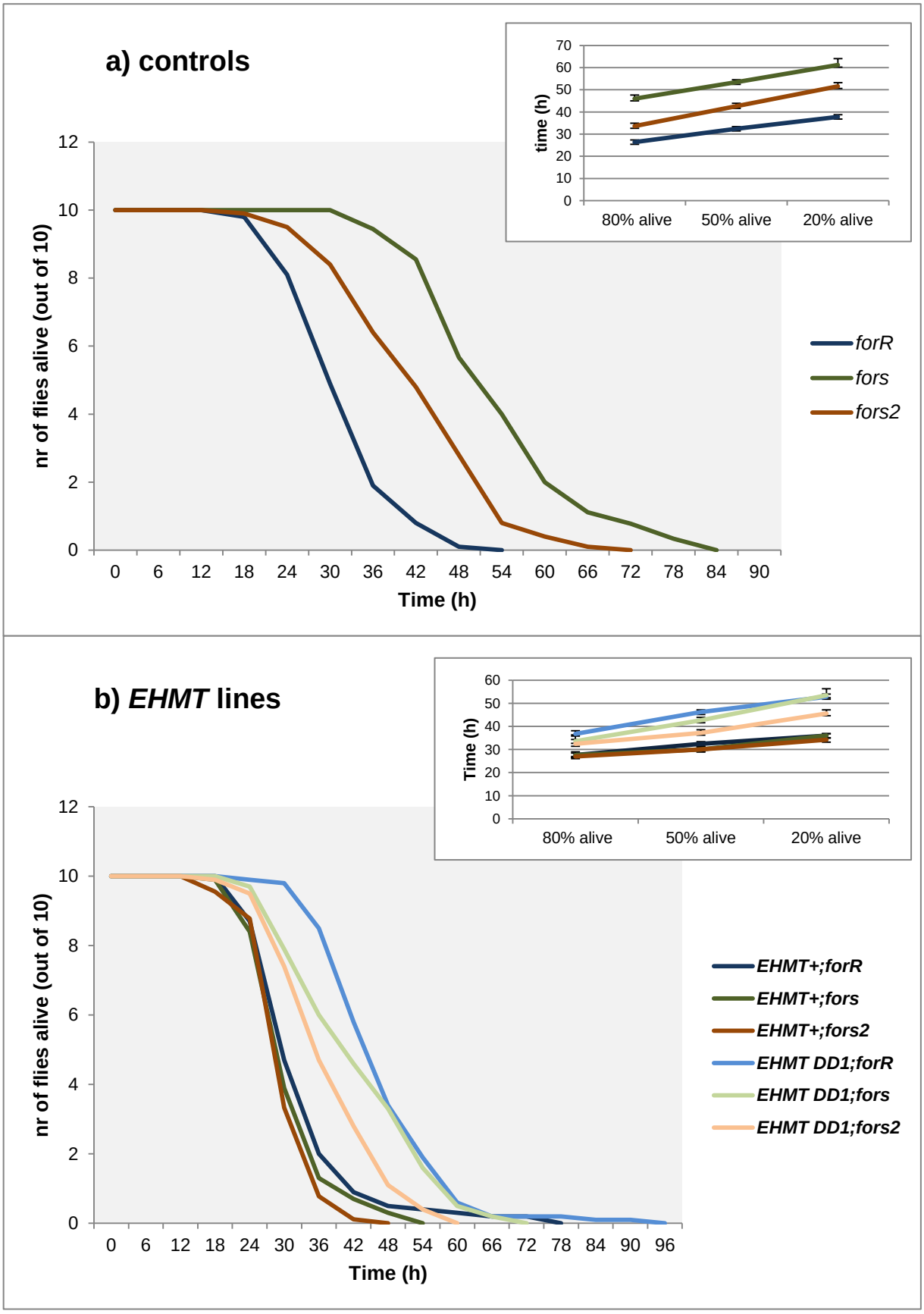


**Figure 3.5.2 – Survival rates for male flies of all strains, demonstrating a) $for^{R}$, $for^{S}$ and $for^{s2}$ control lines and b) $EHMT^{+};for^{R}$, $EHMT^{+};for^{S}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{S}$ and $EHMT^{DD1};for^{s2}$. Inserts show the mean time (h) to 80%, 50% and 20% of flies alive ± standard error.**

## 3.6 – The loss of *EHMT* results in higher triglyceride storage levels

To analyze whether *EHMT* related differences in adult feeding behaviour and survivorship under food deprivation could be related to differences fat storage levels, all strains were tested for weight, triglyceride levels and total protein levels. The mean ± SE for these parameters are shown for all strains in fed and food-deprived conditions in Table 3.1. Although there were no significant differences in weight between the laboratory control strains (one-way ANOVA: $F_{(2)} = 2.622$, $p = 0.082$), the difference between fed and food-deprived flies was significant (one-way ANOVA: $F_{(1)} = 25.583$, $p < 0.001$), the interaction between strains and treatments was not significant (two-way ANOVA: $F_{(2,1)} = 2.197$, $p = 0.121$). In the *EHMT* lines statistical analysis showed significant differences in weight between strains (one-way ANOVA: $F_{(5)} = 4.721$, $p < 0.001$) and treatments (one-way ANOVA: $F_{(1)} = 30.517$, $p < 0.001$), but no significant interaction between strains and treatments (two-way ANOVA: $F_{(5,1)} = 2.265$, $p = 0.053$).

**Table 1.6 - Mean weight (mg), protein and triglycerides of 4 adult females ± SE**

| | **Mean weight ± SE (mg)** | | **Mean Protein ± SE (mg/mL)** | | **Mean Triglycerides ± SE (mg/mL)** | |
|---|---|---|---|---|---|---|
| | Fed | Food deprived | Fed | Food deprived | Fed | Food deprived |
| $for^{R}$ | 5.2 ± 0.42 | 4.8 ± 0.13 | 101.7 ± 4.42 | 102.6 ± 2.43 | 56.4 ± 3.23 | 17.7 ± 0.71 |
| $for^{s}$ | 5.6 ± 0.16 | 5.1 ± 0.18 | 123.8 ± 4.44 | 111.8 ± 4.18 | 102.4 ± 4.12 | 34.6 ± 1.89 |
| $for^{s2}$ | 5.7 ± 0.15 | 4.7 ± 0.15 | 109.3 ± 5.20 | 123.7 ± 3.63 | 61.1 ± 5.08 | 39.1 ± 2.70 |
| $EHMT^{+};for^{R}$ | 5.4 ± 0.16 | 4.6 ± 0.16 | 109.1 ± 2.74 | 101.9 ± 3.46 | 35.5 ± 1.51 | 21.1 ± 2.41 |
| $EHMT^{+};for^{s}$ | 4.8 ± 0.13 | 4.7 ± 0.15 | 99.6 ± 3.41 | 103.8 ± 2.70 | 46.8 ± 1.79 | 21.4 ± 1.74 |
| $EHMT^{+};for^{s2}$ | 5,1 ± 0,1 | 4.9 ± 0.18 | 100.8 ± 3.04 | 113.9 ± 4.23 | 38.0 ± 1.38 | 22.2 ± 1.28 |
| $EHMT^{DD1};for^{R}$ | 4.5 ± 0.17 | 4.1 ± 0.18 | 120.4 ± 2.81 | 96.4 ± 1.80 | 59.4 ± 3.46 | 39.5 ± 1.87 |
| $EHMT^{DD1};for^{s}$ | 5.2± 0.25 | 4.2 ± 0.13 | 104.4 ± 4.23 | 101.8 ± 3.66 | 68.1± 4.06 | 37.5 ± 1.69 |
| $EHMT^{DD1};for^{s2}$ | 5.1± 0.18 | 4.3 ± 0.16 | 94.7 ± 3.22 | 90.4 ± 4.92 | 71.4 ± 3.63 | 32.8 ± 2.69 |

Total protein levels were similar for all strains and/or treatments. In contrast, there were obvious differences of total triglyceride levels between strains and/or treatments. These results were utilized to calculate the triglyceride-protein ratio for further analysis. The mean triglyceride-protein ratio plus or minus standard errors for all strains and treatments are shown in Figure 3.6. These were 0.53 ± 0.02 for $for^{R}$ , 0.83 ± 0.03 for $for^{S}$ , 0.55 ± 0.03 for $for^{s2}$, 0.33 ± 0.01 for $EHMT^{+};for^{R}$, 0.47 ± 0.02 for $EHMT^{+};for^{S}$, 0.38 ± 0.02 for $EHMT^{+};for^{s2}$, 0.5 ± 0.03 for $EHMT^{DD1};for^{R}$, 0.66 ± 0.04 for $EHMT^{DD1};for^{S}$ and 0.76 ± 0.04 for $EHMT^{DD1};for^{s2}$ in fed animals. The values for food-deprived animals were 0.17 ± 0.01 for $for^{R}$, 0.32 ± 0.02 for $for^{S}$, 0.32 ± 0.02 for $for^{s2}$, 0.21 ± 0.03 for $EHMT^{+};for^{R}$ , 0.21 ± 0.02 for $EHMT^{+};for^{S}$ , 0.2 ± 0.01 for $EHMT^{+};for^{s2}$, 0.41 ± 0.02 for $EHMT^{DD1};for^{R}$, 0.37 ± 0.02 for $EHMT^{DD1};for^{S}$ and 0.37 ± 0.03 for $EHMT^{DD1};for^{s2}$. Statistical analysis for the laboratory control showed that there were significant differences between strains (one-way ANOVA: $F_{(2)} = 40.139$, $p < 0.001$), treatments (one-way ANOVA: $F_{(1)} = 337.124$, $p < 0.001$) as well as strain by treatment interactions (two-way ANOVA: $F_{(2,1)} = 15.586$, $p < 0.001$). The same was the case for the *EHMT* strains, with significant differences between strains (one-way ANOVA: $F_{(5)} = 41.298$, $p < 0.001$), treatments (one-way ANOVA: $F_{(1)} = 197.676$, $p < 0.001$) as well as strain by treatment interactions (two-way ANOVA: $F_{(5,1)} = 8.658$, $p < 0.001$).

In regards to control laboratory lines, post hoc multiple comparison procedures showed that fed control rovers have significantly lower triglyceride levels than sitters (Holm-Sidak method: $t = 8.445$, $p < 0.001$). Curiously, $for^{s2}$ fat levels are not significantly different from rovers (Holm-Sidak method: $t = 0.564$, $p = 0.575$) and significantly lower than sitters (Holm-Sidak method: $t = 7.880$, $p < 0.001$). The 24 h food deprivation period resulted in significant lower triglyceride levels in all control strains (Holm-Sidak method: $t = 10.324$, $p < 0.001$ for $for^{R}$, $t = 14.680$, $p < 0.001$ for $for^{S}$ and $t = 6.798$, $p < 0.001$ for $for^{s2}$). Similarly to fed flies, food-deprived rovers showed significantly lower levels of lipids than sitters (Holm-Sidak method: $t = 4.089$, $p < 0.001$). However, contrary to fed conditions, after food-deprivation $for^{s2}$ flies had significantly lower triglyceride levels from rovers (Holm-Sidak method: $t = 4.090$, $p < 0.001$) but not from sitters (Holm-Sidak method: $t = 0.000885$, $p = 0.999$).

The fed *EHMT* revertant lines exhibited similar macronutrient profiles as the wild type laboratory controls, with significant higher triglyceride levels in sitters than in rovers (Holm-Sidak method: $t = 3.806$, $p = 0.001$) and $for^{s2}$ showing intermediate values which were not significantly different to rovers or sitters (Holm-Sidak method: $t = 1.406$, $p = 0.299$ and $t = 2.400$, $p = 0.053$, respectively). The rover-sitter differences in fat storage were maintained in the *EHMT* mutant lines, with rovers having significantly lower triglyceride levels than sitters (Holm-Sidak method: $t = 4.101$, $p < 0.001$). *EHMT* mutant $for^{s2}$ flies had significant higher levels than rover (Holm-Sidak method: $t = 6.768$, $p < 0.001$) and sitter flies (Holm-Sidak method: $t = 2.667$, $p = 0.035$).

As observed in the laboratory controls, there was a significant reduction in lipid levels after 24 h food-deprivation in all *EHMT* mutant and revertant strains. Although this reduction was significant in all cases, it was less accentuated in strains with the *foraging* rover allele (Holm-Sidak method: $t = 3.034$, $p = 0.003$ for $EHMT^{+};for^{R}$ and $t = 2.271$, $p = 0.021$ for $EHMT^{DD1};for^{R}$) than in strains with the sitter (Holm-Sidak method: $t = 6.910$, $p < 0.001$ for $EHMT^{+};for^{S}$ and $t = 7.441$, $p < 0.001$ for $EHMT^{DD1};for^{S}$) or $for^{s2}$ alleles (Holm-Sidak method: $t = 4.725$, $p < 0.001$ for $EHMT^{+};for^{s2}$ and $t = 10.058$, $p < 0.001$ for

*EHMT*$^{DD1}$;*for*$^{s2}$). Interestingly, the differences in fat storage levels between rovers, sitters and *for*$^{s2}$ disappeared upon food-deprivation (see S4 for multiple comparison values).

Importantly, the absence of functional *EHMT* resulted in a highly significant increase in fat-storage levels in all 2$^{nd}$ chromosome backgrounds. This was true for both fed and food-deprived animals (see S4 for multiple comparison values) and suggests that *EHMT* plays a role in lipid storage levels regulation, limiting the amount of fat flies accumulate.

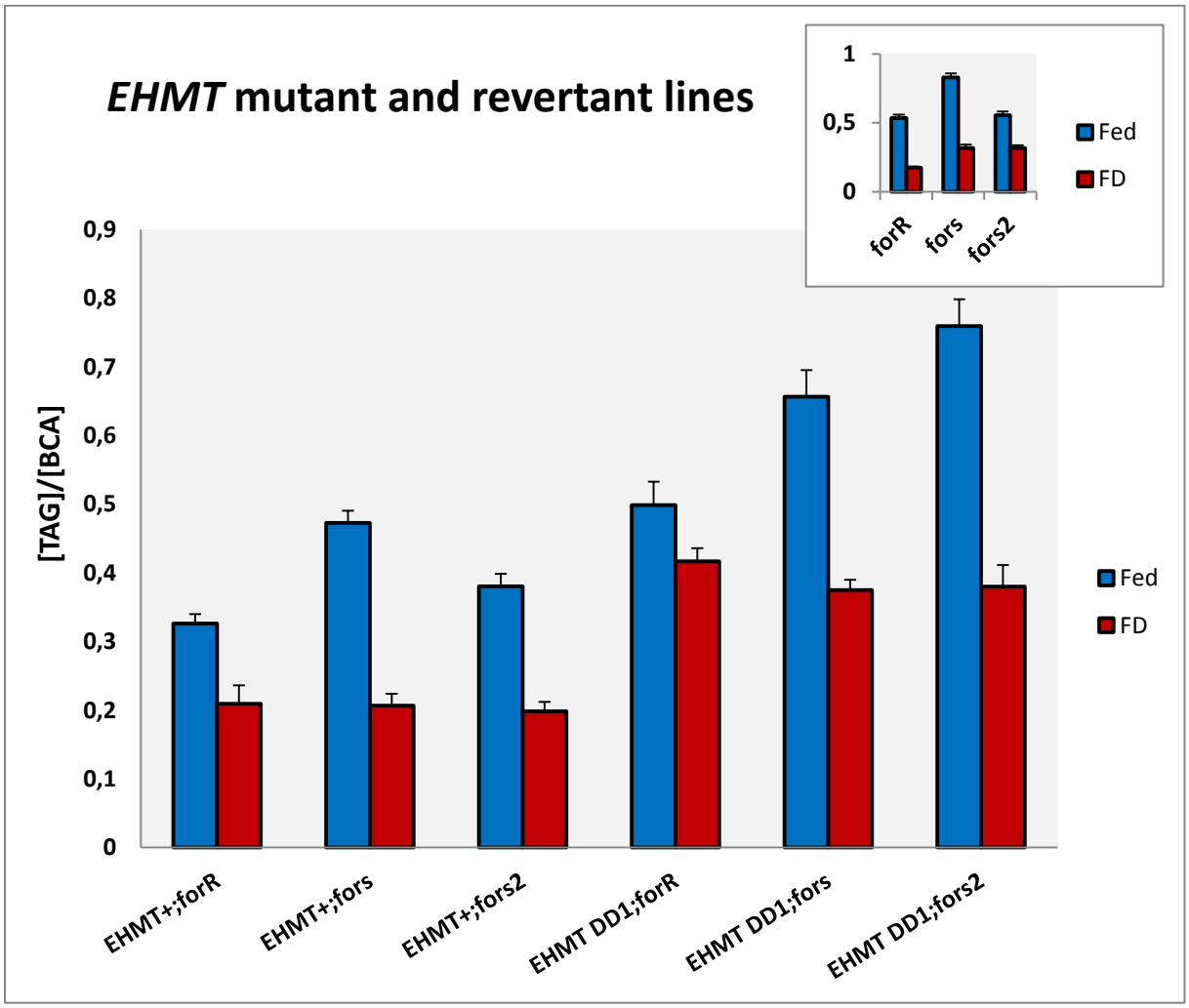


**Figure 3.6 – Mean ± SE triglyceride-protein ratio of 10 biological replicates for all strains. Fed and FD stand for fed and food deprived conditions. Inlet shows the laboratory control strains.**

## 3.7 – Quantitative Real-Time PCR did not detect major differences in for gene expression associated to *EHMT*

To assess if *EHMT* affects *foraging* transcription, qRT-PCR was performed on whole-body total RNA extractions from rover, sitter and $for^{s2}$ Sokolowski Lab lines as well as *EHMT* revertant and mutant adult flies. Three biological replicates were analyzed for each strain using the expression data from *actin5c* and *α-tubulin* as reference genes. *Rp49* was not used in the analysis as it proved to be highly expressed in all strains, evident as very low threshold cycle values.

For analysis of *for* expression in the Sokolowski Lab control lines, standardized expression values for $for^{s}$ were used to calculate fold changes in expression of the other two strains. As shown in Figure 3.7.1 the three primer combinations used for *for* yielded different results, with only q3long primers showing no significant differences between strains and/or treatments. To analyze differences in gene expression between strains and/or feeding regimes, standardized $C_t$ ($\Delta C_t = {}_{Ct}$ gene – mean $C_t$ *actin5c* and *α-tubulin*) were utilized. Analysis for qPcom showed significant differences between strains (one-way ANOVA: $F_{(2)} = 5.232$, $p = 0.011$) and feeding regimes (one-way ANOVA: $F_{(1)} = 4.367$, $p = 0.045$) with no significant strain by treatment interactions (two-way ANOVA: $F_{(2,1)} = 0.286$, $p = 0.753$). Post-hoc tests showed that rovers had significantly higher *for* RNA expression than sitters (Holm-Sidak Method: DM = 1.135; t = 3.120; $p < 0.001$) and that $for^{s2}$ had the same expression levels as rovers. The largest differences were seen with q3short primer pair with highly significant differences between strains (one-way ANOVA: $F_{(2)} = 10.914$, $p < 0.001$) and feeding regimes (one-way ANOVA: $F_{(1)} = 13.833$, $p = p < 0.001$) and no significant strain by treatment interactions (two-way ANOVA: $F_{(2,1)} = 0.481$, $p = 0.623$). As qPcom, q3short showed a high up-regulation of RNA expression in $for^{R}$ and $for^{s2}$ comparing to $for^{s}$ (Holm-Sidak Method: DM = 1.478; t = 4.406; $p < 0.001$ for $for^{R}$ and DM = 1.263; t = 3.619; p = 0.002 for $for^{s2}$). The differences seen with qPcom and q3short were true for both fed and food-deprived animals, although larger in fed flies.

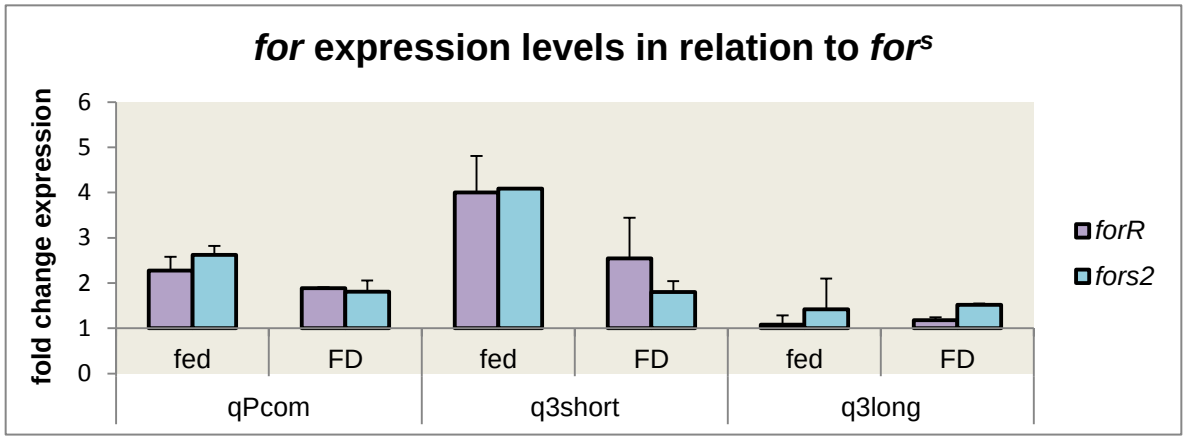


**Figure 3.7.1 - For RNA expression levels for fed and food-deprived $for^{R}$ and $for^{s2}$ using α-tubulin and actin5c as reference genes and in comparison to $for^{s}$ expression levels. Data presented for 3 *foraging* primer pairs: qPcom, q3short and q3long.**

Contrary to what was observed with the control lines, the results obtained with the *EHMT* lines showed large variations in *foraging* gene expression between biological replicates, as shown in Figures 3.7.2 and 3.7.3. In order to examine *for* expression in these lines, the data was analyzed in two ways. The first analysis involved using standardized $C_t$ values obtained for $EHMT^{+};for^{S}$ to calculate comparative fold changes in all other strains. Although ANOVAs showed no differences between the fed *EHMT* revertants strains, the results for these strains suggest similar *for* expression patterns as the laboratory control lines (i.e. rovers and $for^{s2}$ expressing more *for* RNA than sitters, being the largest difference seen with the q3short primer pair) as shown in Figure 3.7.7. Interestingly, conversely to the control lines, *EHMT* mutants all showed the same expression levels regardless of the *foraging* allele. Furthermore, no differences were observed between strains in food-deprived *EHMT* revertant or mutant flies.

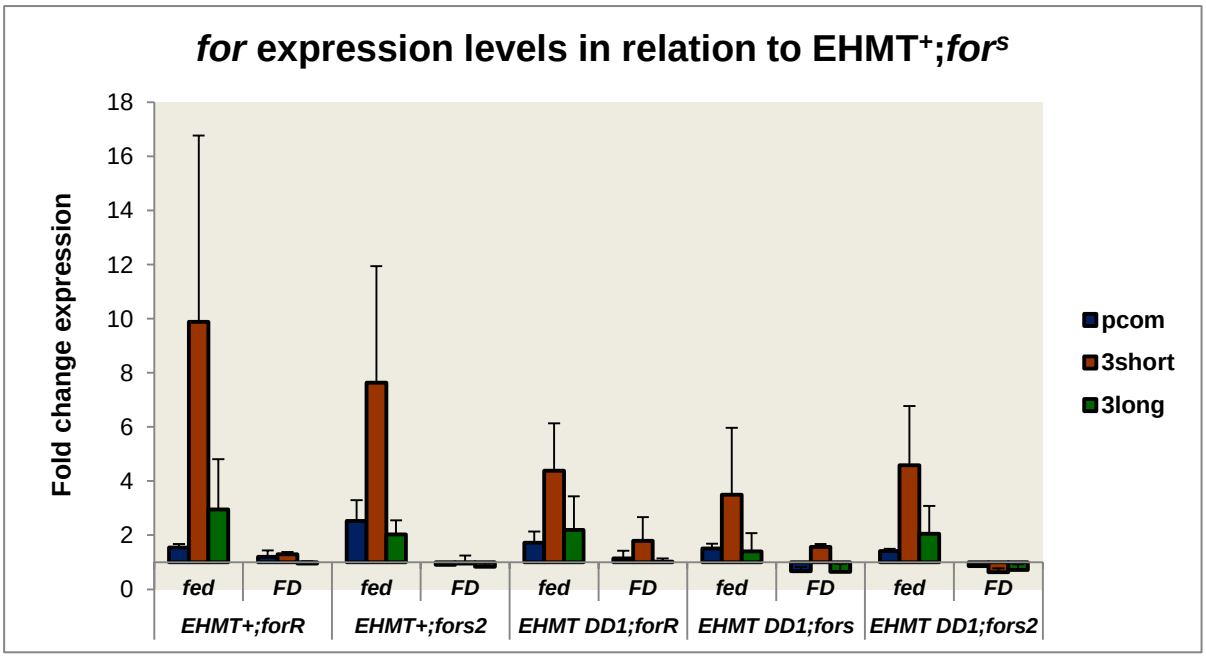


**Figure 3.7.2 - For RNA expression levels for fed and food-deprived $EHMT^{+};for^{R}$, $EHMT^{+};for^{s2}$, $EHMT^{DD1};for^{R}$, $EHMT^{DD1};for^{s}$ and $EHMT^{DD1};for^{s2}$ using α-tubulin and actin5c as reference genes and in comparison to $EHMT^{+};for^{S}$ expression levels. Data presented for all 3 for-primer pairs: qPcom, q3short and q3long.**

*EHMT* mutants in all 2nd chromosome backgrounds were also compared to their respective *EHMT* revertants. In this second analysis, fold changes in expression of the mutants were calculated using the standardized $C_t$ values obtained for the revertants ($EHMT^{DD1};for^{R}$ vs $EHMT^{+};for^{R}$, $EHMT^{DD1};for^{S}$ vs $EHMT^{+};for^{S}$ and $EHMT^{DD1};for^{S2}$ vs $EHMT^{+};for^{S2}$ ). The results are illustrated in Figure 3.7.8, and show no significant differences in *for* gene expression between *EHMT* mutants and revertants, regardless of *for* genetic background.

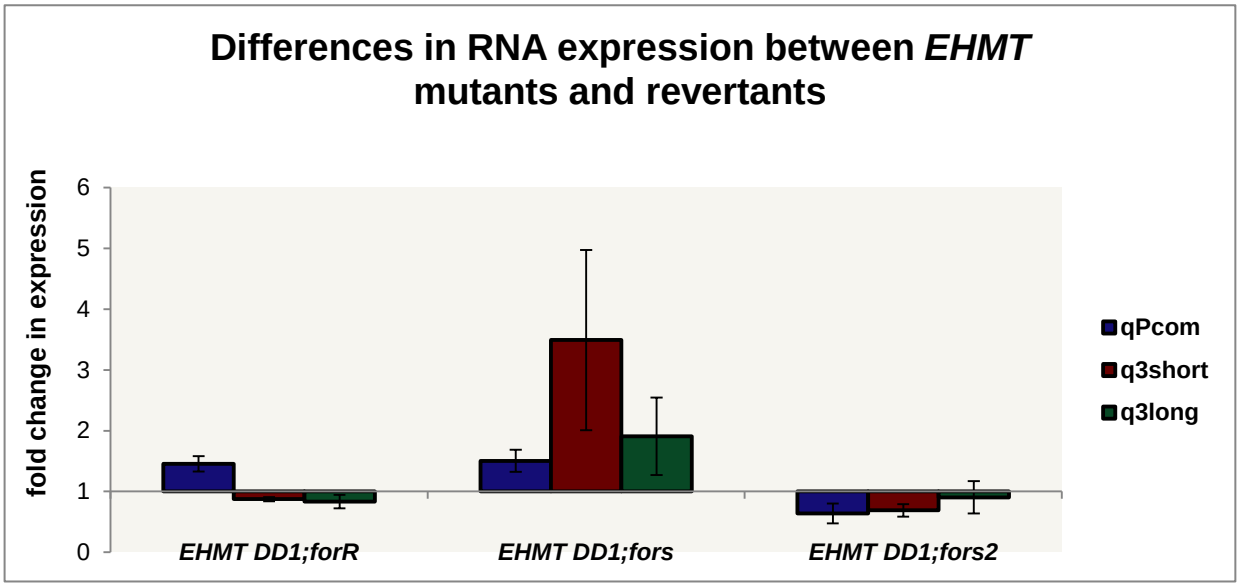


**Figure 3.7.3 – For RNA expression levels for fed $EHMT^{DD1};for^{R}$ , $EHMT^{DD1};for^{S}$ and $EHMT^{DD1};for^{S2}$ comparing to and $EHMT^{+};for^{R}$ , $EHMT^{+};for^{S}$ $EHMT^{+};for^{S2}$ respectively. CTs equilibrated to α-tubulin and actin5c. Data presented for all 3 for-primer pairs: qPcom, q3short and q3long.**

## 3.8 –Variations in FOR protein expression between strains and feeding regimes

FOR protein expression was analyzed by western blotting utilizing 3 biological replicates for each of the *EHMT*$^{+}$*;for*$^{R}$, *EHMT*$^{+}$*;for*$^{S}$, *EHMT*$^{DD1}$*;for*$^{R}$ and *EHMT*$^{DD1}$*;for*$^{S}$ strains and the concentrations of actin as loading control. The actin and FOR bands on the western blots were quantified for density (i.e. intensity), using Fiji from ImageJ. Interestingly, visual analysis of the western blots shows differences between the FOR isoforms detected. As can be seen in figure 3.8, the FOR western blots show 5 bands: band #1 and #3 have been previously identified in the Sokolowski Lab as belonging to the FOR isoform P1, band #4 corresponds to the isoform P3 and band #2 was described as unspecific binding of the antibody used. Band #5 has been detected previously with this antibody, but has not been associated to a specific FOR isoform (Amsale Belay, personal communication).

To analyze total FOR expression, the intensity of all FOR bands (corresponding to all isoforms) was added and the sum was divided by the intensity of the actin band for each sample. To calculate the relative differences between strains and/or treatments, the intensity of fed *EHMT*$^{+}$*;for*$^{S}$ flies was used as reference and therefore the intensities of all other samples were divided by the intensity of *EHMT*$^{+}$*;for*$^{S}$. Mean relative values ± Standard Error of total FOR density are shown in Table 3.8. Statistical analysis showed significant differences between strains (one-way ANOVA: $F_{(3)}$= 3.946, p = 0.028) and between fed and food-deprived treatments (one-way ANOVA: $F_{(1)}$= 4.762, p = 0.044) but no significant strain by treatment interactions (two-way ANOVA: $F_{(3,1)}$= 0.510, p = 0.681). Further analysis involving post-hoc pairwise comparisons showed that the difference between the strains can be attributed to flies with the sitter 2$^{nd}$ chromosome, evident as *EHMT*$^{+}$*;for*$^{S}$ expressing significantly less FOR than *EHMT*$^{DD1}$*;for*$^{S}$ (Holm-Sidak method: t = 3.338, p = 0.025). In general flies with the rover 2$^{nd}$ chromosome show higher total FOR levels than flies with the sitter 2$^{nd}$ chromosome, although these differences were not statistically significant.

**Table 3.8 – Relative differences in FOR protein concentration using *EHMT*$^{+}$*;for*$^{S}$ as reference and actin as loading control.**

| Strain | Treatment | Replicate 1 | Replicate 2 | Replicate 3 | Mean ± SE |
|---|---|---|---|---|---|
| ***EHMT*$^{+}$*;for*$^{R}$** | Fed | 1.066 | 1.795 | 1.602 | 1.488 ± 0.218 |
| ***EHMT*$^{+}$*;for*$^{R}$** | FD | 1.306 | 2.231 | 1.284 | 1.607 ± 0.312 |
| ***EHMT*$^{+}$*;for*$^{S}$** | Fed | - | - | - | - |
| ***EHMT*$^{+}$*;for*$^{S}$** | FD | 1.200 | 2.211 | 1.022 | 1.478 ± 0.370 |
| ***EHMT*$^{DD1}$*;for*$^{R}$** | Fed | 1.436 | 1.323 | 1.345 | 1.368 ± 0.034 |
| ***EHMT*$^{DD1}$*;for*$^{R}$** | FD | 1.209 | 2.509 | 1.411 | 1.710 ± 0.404 |
| ***EHMT*$^{DD1}$*;for*$^{S}$** | Fed | 1.561 | 2.091 | 1.685 | 1.779 ± 0.160 |
| ***EHMT*$^{DD1}$*;for*$^{S}$** | FD | 2.100 | 3.377 | 2.256 | 2.578 ± 0.402 |

As previously stated, there were also significant differences between feeding regimes. Interestingly, these differences were quantitative as well as qualitative, suggesting that feeding regimes differentially effect the expression of specific FOR isoforms. As seen in figure 3.8, although bands #1, #3 and #4 are present in all strains and treatments, band #3 (equivalent to FOR P1) seems to be more intense in food-deprived than in fed flies for all strains. To further analyze this difference, the intensities of band #3 relative to actin were compared statistically and the means ± SE were: 0.84 ± 0.232 for $EHMT^{+};for^{R}$, 0.55 ± 0.191 for $EHMT^{+};for^{S}$, 0.46 ± 0.088 for $EHMT^{DD1};for^{R}$ and 0.35 ± 0.1 $EHMT^{DD1};for^{S}$ for fed animals and 1.46 ± 0.280 for $EHMT^{+};for^{R}$, 0.95 ± 0.180for $EHMT^{+};for^{S}$, 0.79 ± 0,185 for $EHMT^{DD1};for^{R}$ and 0.92 ± 0.262 for $EHMT^{DD1};for^{S}$ for food-deprived flies. These values showed that fed flies have significantly lower expression of the FOR P1 isoform than food-deprived flies (one-way ANOVA: $F_{(1)}$= 10.719, p = 0.005) and this was true for all strains (Holm-Sidak method: t = 3.274, p = 0.005).

Furthermore, there are clear differences in band #5 between strains and treatments. In the *EHMT* revertants this band is almost absent in food-deprived flies, while clearly discernible in fed flies. In the *EHMT* mutants, band #5 is almost absent in fed and completely absent in food-deprived flies with the $for^{R}$ 2nd chromosome, but clearly visible in both fed and food-deprived flies with the $for^{S}$ 2nd chromosome, although fainter in the food-deprived flies. This suggests that the expression of the isoform corresponding to this band is influenced by food-deprivation as well as by *EHMT*, and that this effect is different in rovers and sitters.

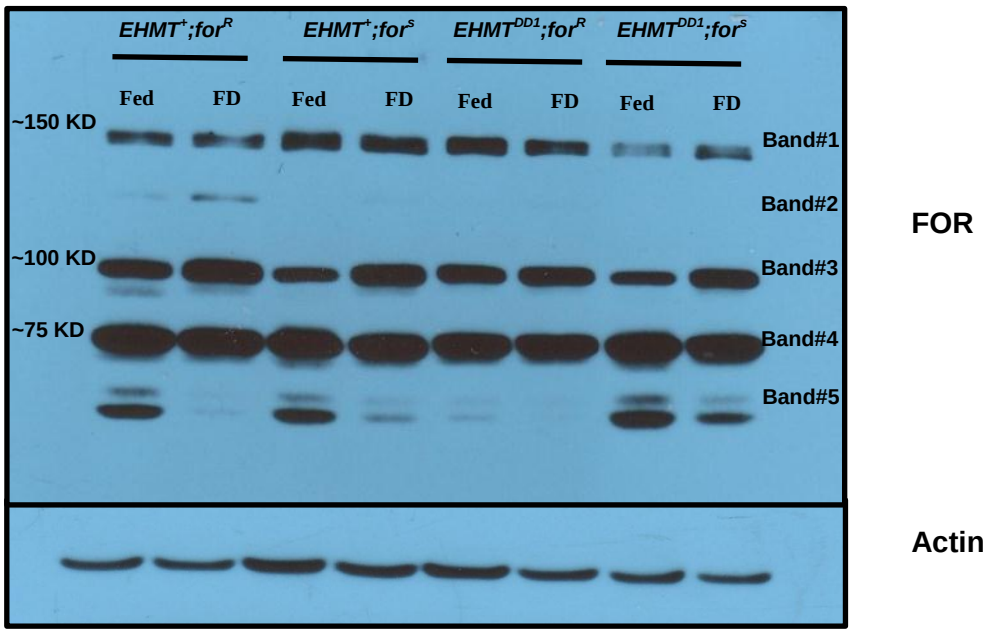


**Figure 3.8 – Western blot for Fed and Food-deprived (FD) flies of $EHMT^{+};for^{R}$, $EHMT^{+};for^{S}$, $EHMT^{DD1};for^{R}$ and $EHMT^{DD1};for^{S}$ strains where: Band#1 and Band#3 correspond to FOR isoform P1; Band#4 corresponds to isoform P3; Band#2 originates from unspecific binding of the antibody and Band#5 corresponds to a unidentified FOR isoform**

# 4 – Discussion

## The role of *EHMT*-mediated epigenetic regulation in larval feeding behaviour

In an ever changing environment, the ability of adapting to new conditions is essential for fitness and survival. The fact that organisms adjust their behaviour in response to environmental conditions is not a novelty, and evolutionary biology has since long been trying to understand the mechanisms that underlie phenotypic plasticity. There are many factors that influence behaviour, involving the balance of positive and negative effects of a particular phenotypic trait on fitness. The main question remains on understanding how environmental stimuli are processed and give rise to a specific behavioural response.

Larval feeding strategies play an essential role in the rate of larval development (Sewell and Connolly, 1975). The feeding rate over successive phases of larval growth determines the time needed to achieve the critical mass needed for pupation and therefore the duration of the larval period as well as the size of adult flies (Bakker, 1961; 1969). Therefore it is not surprising that differences in larval feeding behaviour have been directly linked to overall egg-to-adult viability (Ohnishi, 1979).

The rover and sitter strategies observed in *D. melanogaster* larva exist in nature as a balanced behavioural polymorphism that is characterized by two distinct feeding strategies mainly involving the locomotory rates while foraging. For this polymorphism to be maintained in natural populations, both strategies need to present physiological advantages. In a natural environment where the availability of food is not constant, the sitter strategy might provide an advantage when food is distributed continuously, since moving less involves less energy output, but when food is distributed discontinuously rovers would have the advantage of finding more food due to the expanded search area. Thus, each strategy would provide a larva with a competitive advantage when the environment is consistent with the strategy (Sokolowski, 1983).

It has been previously described that these phenotypes in *D. melanogaster* larvae are plastic when exposed to varying environmental parameters, evident as the rover-sitter polymorphism being only expressed on a food substrate (Sokolowski *et al*, 1983) and acute starvation inducing changes in this trait, as food deprived larvae move significantly less than fed larvae (Graf and Sokolowski, 1989).

In addition, rovers show lower food intake and higher glucose absorption than sitters and *for*$^{s2}$ when food is plentiful, but chronic food deprivation results in a plastic response in this behaviour, with all larvae increasing food intake to a common maximal level (Kaun *et al*, 2007). Acute food-deprivation results in decreased hemolymph sugar levels in rovers but not in sitters (Kaun et al, 2008). However, the mechanisms that permit larvae to express this behavioural plasticity are still unknown. In this work, I show how EHMT mediated histone methylation at the *foraging* promoters epigenetically mediates the plasticity seen in larval *foraging*-related traits.

Results showed that acute food deprivation results in a significant reduction in path lengths while foraging in all rover and sitter and *for*$^{s2}$ flies, as expected. Although there was no effect of *EHMT* in the first larval path length assay, when the 2$^{nd}$ and 3$^{rd}$ chromosomes were not controlled, with the second larval path length assay, where the strains were generated with controlled *for*$^{R}$, *for*$^{s}$ or *for*$^{s2}$ 2$^{nd}$ chromosomes, I showed here for the first time that the loss of functional EHMT affects the plastic response to starvation. While larvae with functional EHMT respond to food deprivation by reducing the locomotory rate while feeding, larvae without functional EHMT have a much lower response to food deprivation in this trait. Furthermore, this study revealed that the effects of *EHMT* are only seen under food-deprivation, evident as fed larvae with *EHMT* exhibit the same phenotypes as fed larvae without *EHMT*, but starved larva without *EHMT* show significantly higher path lengths than starved larva that express functional *EHMT*. Consequently, starved *EHMT* mutants show locomotory rates almost as high as fed mutants, demonstrating that functional *EHMT* does not significantly affect larval feeding behaviour in well-nourished animals but is needed for the natural response to food-deprivation. Reducing the locomotory rate while feeding after food-deprivation, might have a physiological advantage since less energy is expended on moving.

The fact that the observed effect of *EHMT* is the same on rover, sitter and *for*$^{s2}$ flies indicates that epigenetic regulation through *EHMT* methylation is not what underlies the rover-sitter polymorphism in larval feeding patterns.

## Importance of *EHMT* in expression of rover - sitter differences in adult foraging behaviour

Previous studies have shown that there are significant rover - sitter differences in adult foraging behaviour when food is present. Adult rover and sitter flies differ in locomotory patterns after feeding on a sucrose drop, with rovers moving farther away from the food source than sitters (Nagle and Bell, 1987; Pereira and Sokolowski, 1993). As in larval feeding patterns, this polymorphism might represent adaptations to different environments. Accordingly, rovers may be better adapted to exploit environments where food is distributed in patches, whereas sitters may be better able to exploit environments where food is homogeneously distributed, without wasting energy in locomotion. It has been shown that this polymorphism originates at the *foraging* locus, but the molecular mechanisms underlying it are still unclear.

Adult feeding behaviour is influenced by many variables, and changes in feeding regime or nutritional state affect traits such as ingestion responsiveness and meal volume. Flies fed *ad libitum* maintain smaller crop volumes than food-deprived flies, and respond differently to sucrose solutions (Edgecomb *et al*, 1994). Consequently, some behavioural polymorphisms are only expressed under certain environmental conditions. For instance, food-deprived rover and sitter adults show differences in food-leaving scores after being offered a sucrose meal. While sitters leave the area after the meal, rovers tend to stay in the area. This difference is only seen in food-deprived flies, since fed rovers and sitters exhibit equally high food leaving scores (Kent *et al*, 2009). Additionally, 24 h food-deprived rovers are more responsive to sucrose than sitters (Scheiner *et al*, 2004).

Previous unpublished tests performed by Bryon Hughson in the Sokolowski lab showed that fed adult rover and sitter flies tested in the adult foraging assay used in this thesis do not differ in their behaviour, but when food deprived prior to the test, rovers show more intense localized food search behaviour and consume more sucrose drops than sitters. My results agree with his findings, with 24 h food deprived adult rover females consuming significantly more sucrose drops than sitter and $for^{s2}$ flies. This suggests that starvation affects rovers and sitters differently, with rovers showing a higher response to food-deprivation. Interestingly my results show that *EHMT* is needed for the expression of this behavioural polymorphism, and when present *EHMT* seems to down regulate the response to food deprivation in sitters. This is evident as rover flies expressing *EHMT* exhibit the same intense food-searching and sucrose drop consumption as rovers without functional *EHMT*; but sitter mutants lacking *EHMT* have much higher levels of sucrose drop consumption than sitters expressing *EHMT,* showing the same consumption scores as rovers. Taken together, these results suggest an epigenetic interaction between *EHMT* and the *foraging* alleles that is dependent on environmental parameters.

## Effects of *EHMT* on fat storage levels and starvation resistance in adult flies

The ability to survive in an environment without food (starvation resistance) has been positively correlated with fat storage levels (i.e. body lipid proportion) in *Drosophila* (Ballard *et al*, 2008).

As in so many other traits, *D. melanogaster* strains with the rover and sitter *foraging* alleles differ in the amount of fat storage they accumulate during adulthood as well as the time they are able to survive under acute food-deprivation. Previous studies showed that adult sitters have higher starvation resistance than rovers, surviving longer when no food is available (Donlea *et al*, 2012).

My results corroborate these findings, as sitters survived significantly longer than rovers in my starvation resistance assay. The fact that the survival rates for these strains show similar curves suggests that this is the result of sitters withstanding a longer initial period of starvation. Once the critical point for survival is achieved and flies start to die, sitters die at the same rate as rovers. This can be explained by my results for the triglyceride measurements. Results show that sitters have significantly higher triglyceride levels than rovers when they are well fed, but that after 24h of food-deprivation rover and sitter triglyceride levels drop to almost the same level. Accordingly sitters are

able to survive longer initially, but when the excess of fat storage is depleted; they show the same response to starvation as rovers. An intermediate phenotype between rovers and sitters was observed for $for^{s2}$ mutants, both in starvation resistance and triglyceride storage levels; $for^{s2}$ is an induced mutation (de Belle *et al*, 1989; Pereira and Sokolowski, 1993) compared to $for^{s}$ which is a natural variant, so it is not surprising that these sitter alleles might have different effects on *for*'s pleiotropic phenotypes and this is born out by the results in my thesis for several phenotypes where $for^{s2}$ differs from $for^{s}$. In addition differences between $for^{s}$ and $for^{s2}$ might be due to genetic background effects, since there are often significant interactions between the genetic background and *for*-related phenotypes in traits where rovers and sitters differ in plasticity (Kent *et al*, 2009). Nevertheless the same correlation between lipid levels and starvation resistance can be seen in all *foraging* backgrounds.

These findings also correlate well with my results for the adult foraging assay, as the lower scores of sucrose drop consumption observed in sitters and $for^{s2}$ might be a consequence of these strains being physiologically less affected by the 24 h of food-deprivation to which they were subjected prior to the testing. Hence, if sitters have higher lipid storage levels than rovers, they might be less "hungry" after 24 h without feeding than rovers.

Interestingly, I found that *EHMT* affects both lipid storage levels and resistance to starvation in adult females. The loss of *EHMT* causes a significant increase in fat storage in all the strains tested, as well as significant higher survivorship under starvation. This suggests that *EHMT* methylation plays a role in weight regulation, preventing adult flies from accumulating too much fat. Although having higher energy stores might present an advantage under certain conditions (e.g. acute food-deprivation) in general too much fat is associated with serious health problems. Lipid intake, synthesis, catabolism, and storage as fat are regulated in response to the body's energy demands and when this natural energy homeostasis is in disequilibrium it might severely affect fitness and survival. For instance, obesity in flies has been associated to phenotypes parallel to diabetes (Rulifson *et al*, 2002) and cardiac problems (Wessells *et al*, 2004) as well as to a reduced lifespan (Skorupa *et al*, 2008).

The effect of *EHMT* on body fat regulation seems to be independent of the *foraging* background, since rovers and sitters show an equivalent increase in triglyceride levels in the *EHMT* mutant background, which can be correlated to the increase in starvation resistance observed. As a consequence, the rover-sitter differences observed in these phenotypes with laboratory control strains are not affected by the loss of *EHMT.* Nonetheless, the increase in starvation resistance seems to be larger in sitters than in rovers, although the increase in lipid levels is the same in both variants. Curiously, $for^{s2}$ *EHMT* mutant females show the highest triglyceride storage levels but have less starvation resistance than rover and sitter *EHMT* mutant females. This might be due to the fact that $EHMT^{DD1};for^{s2}$ is a double mutant and consequently less healthy than the $EHMT^{DD1};for^{R}$ and $EHMT^{DD1};for^{s}$ which carry the *for* natural variants.

After 24 h of food-deprivation the rover, sitter and $for^{s2}$ *EHMT* mutants still maintain higher triglyceride levels in respect to the respective revertants, showing again the correlation with increased starvation resistance. Taken together, my results show that both *foraging* and *EHMT* affect energy

storage levels and starvation resistance, but an obvious interaction between the two genes could not be found in these phenotypes.

## Effects of *EHMT* on *for* RNA and protein expression

Since no discrete differences have been found in the nucleotidic sequence of the rover and sitter *for* alleles, it seems close at hand that the behavioural polymorphisms related to this gene might arise from differential expression of the gene. If that is the case, the obvious question following would be which mechanism(s) mediate the differential expression of *for* in rovers and sitters and if those mechanisms are also involved in the plasticity seen in *for*-related behaviours. Previous work has shown differences in *for* RNA expression levels in rover, sitter and $for^{s2}$ flies. RNA extractions from adult heads, analyzed by Northern analysis revealed that $for^{s}$ and $for^{s2}$ express slightly less (about 10%) *for* RNA than $for^{R}$. Three major *for* transcripts were analyzed and although all yielded similar results, with rovers expressing more RNA than sitters and $for^{s2}$, the largest differences were seen in the T1 transcript. The results of that study also suggested that $for^{R}$ adult heads have higher FOR protein levels than $for^{s}$ and $for^{s2}$ but this was not specifically related to any of the FOR isoforms, since no *Drodophila* FOR antibody was available at the time and an antibody to bovine PKG being used instead (Osbourne *et al*, 1997). Interestingly, western blot analysis using an anti-FOR antibody on adult *EHMT* mutant and revertant heads (with unknown *for* alleles) revealed that base-line *for* protein levels are not different in *EHMT* wild type versus mutant strains, but that there was a decrease in FOR protein levels after 24 hours starvation in *EHMT* wilt type flies that could not be seen *EHMT* mutants, suggesting thus that starvation mediated reduction of FOR requires *EHMT* (Jamie Kramer, University of Nijmegen, personal communication).

The results obtained in this project for RNA expression levels using qRT-PCR on whole body total RNA are in accordance with what was seen previously in rover and sitter adult heads, with rovers expressing higher levels of *for* RNA than sitters. Unexpectedly $for^{s2}$ flies showed the same results as rovers and not as sitters. The *for* RNA expression in food-deprived adult flies was analyzed for the first time in this study, and results showed the same rover-sitter differences in expression levels of food-deprived animals. Furthermore, the loss of functional *EHMT* seems to result in the loss of rover-sitter differences, with all *EHMT* mutant strains showing the same transcription levels. *for* RNA expression also seems to be more affected by *EHMT* in fed sitters than in rovers and $for^{s2}$, but considering that none of the observed differences were significant, it is not possible to draw reliable conclusions about whether overall *for* expression levels are responsible for the strain and environment-dependent phenotypic differences reported in this thesis. Keeping in mind that *foraging* is a complex gene with many transcripts and several protein isoforms, the fact that the 3 *for*-specific primer pairs used here yielded different results suggests that they do not all amplify the same transcripts and that there might be differences not in overall expression of *for,* but in the expression of specific transcripts. Hence, although there are no major differences in overall *for* expression in whole adult flies, there might be considerable differences in spatial-temporal distribution of the different *for* transcripts. It would be

interesting to further analyze if *EHMT* mediates differential transcription of the different *for* transcripts and if this can be related to the behavioural polymorphisms and plasticity observed.

Interestingly, although there were no major differences in overall for RNA expression between strains or treatments, western blot analysis with FOR-specific antibodies revealed significant changes in FOR protein expression. The results for the overall expression levels of FOR agree with what was previously shown, as rovers expressed slightly more protein than sitters. Also, food-deprived flies consistently expressed more FOR than fed animals. The loss of *EHMT* seemed to result in an increase in overall FOR levels in sitters but not in rovers which could be related to the fact that adult sitters are more affected in some *for*-related phenotypical traits than rovers. More importantly, major differences were observed in regards to the different FOR isoforms. As shown by banding profiles on the western blots, food-deprivation mainly affects two of the detected isoforms. Isoform P1 is significantly up-regulated in food-deprived animals of all strains, and this up-regulation seems to be independent of *EHMT* as the same results could be seen in *EHMT* mutants and revertants. Feeding regime also affects the lowest molecular weight isoform seen on the gels, and interestingly, expression of this isoform is also affected by *EHMT*. While in fed flies with functional *EHMT* it is highly expressed, in food-deprived flies it is almost completely absent. With this being true for rovers and sitters, it is curious that the loss of *EHMT* affects these two *for* variants in opposite ways. Whereas in flies with the rover background the loss of EHMT results in a down-regulation in expression of this isoform in fed animals, in flies with the sitter background it results in an up-regulation in the food-deprived animals. Consequently, this isoform is almost absent in both fed and food-deprived *EHMT* mutants with the rover 2$^{nd}$ chromosome, while clearly expressed in both fed and food deprived *EHMT* mutants with the sitter 2$^{nd}$ chromosome. Although there does not seem to be a clear correlation between the expression patterns of any of the FOR isoforms and the differences observed in phenotypical traits, these results support the hypothesis that it is the expression of specific FOR isoforms that underlies the behavioural plasticity seen with different feeding regimes and that *EHMT* affects FOR expression in sitters and rovers differently. To completely understand how *EHMT* affects *for*-related phenotypical traits, it would be essential to know which FOR isoform(s) are related to which behaviour.

## Possible synergistic effects of *EHMT* and *for* on viability

Previous studies on *Drosophila EHMT* are not in agreement as to whether *EHMT* is necessary for viability. The first study concerning *EHMT* reported the presence of the functional protein to be necessary for normal development since *EHMT*-RNAi knock out mutants where pupal lethal (Stabell *et al*, 2006). In contrast, two later studies showed that *EHMT* null mutants are viable and develop normally (Seum *et al,* 2007; Kramer *et al*, 2011). In the process of generating *EHMT* mutants homozygous for the 2$^{nd}$ chromosome, I found that *EHMT* mutants homozygous for the rover 2$^{nd}$ chromossome are viable but do not reproduce. These results show that $EHMT^{DD1};for^{R}$ mutants are either sterile or that embryos originating from these flies are embryonically lethal. Since crosses of

*EHMT*$^{DD1}$;*for*$^{R}$ males and females with wild-type rover females and males also did not originate progeny, embryonic lethalithy is unlikely since embryos from these crosses would have a functional copy of *EHMT*, and therefore be viable. Accordingly, the results suggest that the gametes of *EHMT*$^{DD1}$;*for*$^{R}$ mutants are not viable, whereas *EHMT*$^{DD1}$;*for*$^{s}$ and *EHMT*$^{DD1}$;*for*$^{s2}$ do not show these viability problems, meaning that methylation patterns mediated by *EHMT* during gametogenesis are essential for the viability of gametes in a genetic background-dependent manner. This is extremely interesting since it suggest synergistic effects of *EHMT* mutants and *for* on the viability of gametes.

Another possibility is that there are distinct reasons for *EHMT*$^{DD1}$;*for*$^{R}$ females and males to not have progeny. It has been suggested previously that maternal *EHMT* is important for embryonic development, since large amounts of protein are passed from the maternal nursing cells into the fertilized egg (Stabell *et al*, 2006). Accordingly embryos originating from a mother without *EHMT* might not be able to develop. Nevertheless, this would not explain the sterility observed in crosses with *EHMT*$^{DD1}$;*for*$^{R}$ males and wild-type females, as cytoplasmatic contents of the male gamete are usually not of great importance for embryonic development and the progeny of this cross would have cytoplasmic EHMT passed from their mother. One alternative possibility for the male sterility is that some physiological or behavioural process related to courtship and mating is affected in *EHMT*$^{DD1}$;*for*$^{R}$ males, and hence they are not able to procriate.

## Concluding remarks and further perspectives

With the results of this thesis I provide a solid base for the role of epigenetic regulation of *foraging*-related phenotypical plasticity. Across the behavioural and physiological assays it is evident that *EHMT* not only significantly affects several phenotypic traits linked to the *foraging* gene but that *EHMT* is needed for the plastic response to food-deprivation. While larva with functional *EHMT* significantly reduce their path-lengths in response to food-deprivation, larvae lacking *EHMT* show the same behaviour as fed larva, suggesting that they are not capable of responding to environmental cues by adjusting their behaviour. In adult flies, similar results are seen in a genetic background-dependent manner. The loss of functional *EHMT* results in an increase in feeding scores of sitters but not rovers after food-deprivation, evident as sitter adults expressing *EHMT* show lower feeding scores than rovers, but sitters without functional *EHMT* have the same high feeding scores as rovers. The feeding scores of rovers are not altered by the lack of *EHMT* and this suggests an epigenetic interaction between *EHMT* and the *foraging* alleles, with *EHMT* down-regulating sitter but not rover adult foraging activity upon food-deprivation. In addition, triglyceride levels of *EHMT* mutants and revertants show that *EHMT* plays a role in fat storage regulation. *EHMT* mutants have higher fat storage levels and the fact that they survive longer during starvation could be a direct consequence. Finally, *EHMT* mutants and revertants do not differ significantly in overall *foraging* RNA expression in fed and food-deprived feeding regimes, but *EHMT* has a strong influence on the FOR protein isoforms expressed, altering the expression pattern upon food-deprivation. Again this effect of *EHMT* is different in rovers and sitters. Taken together, this thesis provides evidence of epigenetically driven

behavioural plasticity, evident as *EHMT* epigenetically regulates traits influenced by the *foraging* gene and this regulation is linked to environmental cues.

Understanding the complex mechanisms underlying phenotypical plasticity has become a major concern not only in evolutionary biology but also in human medicine and psychology. The ability of predicting phenotypic variation from underlying genotypes and environmental factors would offer major advances in disease diagnosis, as well as preventive and therapeutic measures for many human medical conditions. *EHMT* and *foraging* provide an excellent model to study how epigenetic factors can mediate the interaction between genes and environment.

Future work on the interaction of *EHMT* and *foraging* will certainly provide insight about how *EHMT* affects transcription at the *foraging* locus and if there are differences in *EHMT*-mediated methylation at the *foraging* promoters in rover and sitter flies. Techniques such as chromatin immunoprecipitation coupled to qRT-PCR detection (ChIP-qRT-PCR) with *EHMT*-specific antibodies and *foraging*-specific primers would permit to assess differences in *EHMT* activity at the *foraging* locus in rover and sitter flies. To see if the interaction found between *EHMT* and *foraging* is bidirectional, it would be useful to analyze *EHMT* RNA transcription and protein expression levels in rovers and sitters, since it might be that *EHMT* expression varies in different *foraging* genetic backgrounds. It would also be interesting to extend this analysis to environmental effects other than food-deprivation. Furthermore, since the functions of *EHMT* might be time and tissue-specific, it would be useful to narrow down the effects of *EHMT* on *foraging* and *foraging*-related behaviours by targeting expression to subsets of tissues at different developmental stages. Performing qRT-PCR with specific primers for the different *for* transcripts would help in understanding if the effect of *EHMT* on transcription is transcript specific. Dissecting the roles of the different *for* transcripts and protein isoforms using transgenic flies will also undoubtedly aid in our understanding of the genetic, epigenetic, neuronal and physiological processes which influence behavioural plasticity. Moreover, it would be interesting to address the possible synergistic effects of *EHMT* mutations and *foraging* on fertility.

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# Supplementary Data

## S1 - Kruskal-Wallis One Way Analysis of Variance on Ranks for Larval Path Length experiment 1

| | Fed | | | | Food-deprived | | | |
|---|---|---|---|---|---|---|---|---|
| | **N** | **Median** | **25%** | **75%** | **N** | **Median** | **25%** | **75%** |
| ***for*$^{R}$** | 111 | 9,606 | 7,895 | 10,876 | 118 | 4,519 | 1,036 | 6,346 |
| ***for*$^{s}$** | 117 | 4,834 | 2,740 | 6,115 | 120 | 0,677 | 0,560 | 1,043 |
| ***EHMT*$^{+}$** | 120 | 4,502 | 3,102 | 5,504 | 118 | 0,700 | 0,575 | 1,382 |
| ***EHMT*$^{DD1}$** | 119 | 4,911 | 3,984 | 6,284 | 119 | 1,169 | 0,696 | 2,372 |
| H = 557,227 with 7 degrees of freedom. (P = <0,001) | | | | | | | | |

**All Pairwise Multiple Comparison Procedures (Dunn's Method):**

| **Comparison** | **Diff of Ranks** | **Q** | **P<0,05** |
|---|---|---|---|
| *for*$^{R}$ fed vs *for*$^{S}$ FD | 657,201 | 18,342 | Yes |
| *for*$^{R}$ fed vs *EHMT*$^{+}$ FD | 628,190 | 17,462 | Yes |
| *for*$^{R}$ fed vs *EHMT*$^{DD1}$ FD | 549,992 | 15,319 | Yes |
| *for*$^{R}$ fed vs *for*$^{R}$ FD | 320,347 | 8,905 | Yes |
| *for*$^{R}$ fed vs *EHMT*$^{+}$ Fed | 276,880 | 7,728 | Yes |
| *for*$^{R}$ fed vs *for*$^{S}$ Fed | 273,674 | 7,592 | Yes |
| *for*$^{R}$ fed vs *EHMT*$^{DD1}$ Fed | 239,752 | 6,678 | Yes |
| *for*$^{S}$ Fed vs *for*$^{S}$ FD | 383,526 | 10,850 | Yes |
| *for*$^{S}$ Fed vs *EHMT*$^{+}$ FD | 354,516 | 9,987 | Yes |
| *for*$^{S}$ Fed vs *EHMT*$^{DD1}$ FD | 276,318 | 7,801 | Yes |
| *for*$^{S}$ Fed vs *for*$^{R}$ FD | 46,673 | 1,315 | No |
| *for*$^{S}$ Fed vs *EHMT*$^{+}$ Fed | 3,206 | 0,0907 | No |
| *EHMT*$^{+}$ Fed vs *for*$^{S}$ FD | 380,321 | 10,828 | Yes |
| *EHMT*$^{+}$ Fed vs *EHMT*$^{+}$ FD | 351,310 | 9,960 | Yes |
| *EHMT*$^{+}$ Fed vs *EHMT*$^{DD1}$ FD | 273,112 | 7,759 | Yes |
| *EHMT*$^{+}$ Fed vs *for*$^{R}$ FD | 43,467 | 1,232 | No |
| *EHMT*$^{DD1}$ Fed vs *for*$^{S}$ FD | 417,448 | 11,860 | Yes |
| *EHMT*$^{DD1}$ Fed vs *EHMT*$^{+}$ FD | 388,438 | 10,989 | Yes |
| *EHMT*$^{DD1}$ Fed vs *EHMT*$^{DD1}$ FD | 310,239 | 8,796 | Yes |
| *EHMT*$^{DD1}$ Fed vs *for*$^{R}$ FD | 80,595 | 2,280 | No |
| *EHMT*$^{DD1}$ Fed vs *EHMT*$^{+}$ Fed | 37,128 | 1,055 | No |
| *EHMT*$^{DD1}$ Fed vs *for*$^{S}$ Fed | 33,922 | 0,958 | No |
| *for*$^{R}$ FD vs *for*$^{S}$ FD | 336,854 | 9,550 | Yes |
| *for*$^{R}$ FD vs *EHMT*$^{+}$ FD | 307,843 | 8,691 | Yes |
| *for*$^{R}$ FD vs *EHMT*$^{DD1}$ FD | 229,645 | 6,497 | Yes |
| *EHMT*$^{DD1}$ FD vs *for*$^{S}$ FD | 107,209 | 3,046 | No |
| *EHMT*$^{DD1}$ FD vs *EHMT*$^{+}$ FD | 78,199 | 2,212 | No |
| *EHMT*$^{+}$ FD vs *for*$^{S}$ FD | 29,010 | 0,822 | No |

# **S2** - All Pairwise Multiple Comparison Procedures (Holm-Sidak method) for Larval Path Length Experiment 2

**Comparisons for Fed strains:**

| Comparison | Diff of Means | t | P | P<0,05 |
|---|---|---|---|---|
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 4,043 | 11,847 | <0,001 | Yes |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 4,123 | 11,505 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 3,972 | 9,845 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 4,051 | 9,693 | <0,001 | Yes |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 3,135 | 9,135 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 3,063 | 7,564 | <0,001 | Yes |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{s}$ | 2,060 | 6,462 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{s}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 1,983 | 6,194 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{s}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 2,062 | 6,097 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{s}$ | 1,989 | 5,171 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{s}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 1,074 | 3,336 | 0,004 | Yes |
| *EHMT*$^{+}$; *for*$^{s}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 0,988 | 2,735 | 0,025 | Yes |
| *EHMT*$^{+}$; *for*$^{s}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 0,909 | 2,639 | 0,025 | Yes |
| *EHMT*$^{+}$; *for*$^{S2}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 0,0793 | 0,221 | 0,970 | No |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{R}$ | 0,0714 | 0,178 | 0,859 | No |

**Comparisons for Food-Deprived strains:**

| Comparison | Diff of Means | t | P | P<0,05 |
|---|---|---|---|---|
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 5,185 | 12,508 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 4,913 | 12,010 | <0,001 | Yes |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 3,546 | 10,348 | <0,001 | Yes |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 3,274 | 9,743 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{s}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 3,387 | 9,488 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{s}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 3,114 | 8,884 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs EHMT DD1 S2 | 2,817 | 6,683 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{S2}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 2,368 | 6,634 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{S2}$ vs *EHMT*$^{+}$; *for*$^{s}$ | 2,096 | 5,978 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs EHMT DD1 ee | 1,798 | 4,266 | <0,001 | Yes |
| *EHMT*$^{DD1}$; *for*$^{R}$ vs *EHMT*$^{+}$; *for*$^{R}$ | 1,639 | 4,002 | <0,001 | Yes |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 1,178 | 3,356 | 0,003 | Yes |
| *EHMT*$^{DD1}$; *for*$^{s}$ vs *EHMT*$^{DD1}$; *for*$^{S2}$ | 1,019 | 2,791 | 0,016 | Yes |
| *EHMT*$^{+}$; *for*$^{s}$ vs *EHMT*$^{+}$; *for*$^{S2}$ | 0,272 | 0,796 | 0,671 | No |
| *EHMT*$^{+}$; *for*$^{R}$ vs *EHMT*$^{DD1}$; *for*$^{s}$ | 0,159 | 0,454 | 0,650 | No |

# S3 - All Pairwise Multiple Comparison Procedures (Student-Newman-Keuls Method) for Starvation Resistance Assay

**Comparisons for Females at 20 % alive-Time Point:**

| Comparison | Diff of Ranks | q | P<0,05 |
|---|---|---|---|
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{+}$; for$^{S2}$* | 478,000 | 8,655 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{+}$; for$^{R}$* | 370,000 | 8,026 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* ee vs *EHMT$^{+}$; for$^{S}$* | 249,000 | 6,735 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 215,500 | 7,741 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{DD1}$; for$^{R}$* | 133,500 | 7,136 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 344,500 | 7,473 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{R}$* | 236,500 | 6,397 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{s}$* | 115,500 | 4,149 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 82,000 | 4,383 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{S2}$* | 262,500 | 7,101 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{R}$* | 154,500 | 5,550 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{s}$* | 33,500 | 1,791 | No |
| *EHMT$^{+}$; for$^{s}$* vs *EHMT$^{+}$; for$^{S2}$* | 229,000 | 8,226 | Yes |
| *EHMT$^{+}$; for$^{s}$* vs *EHMT$^{+}$; for$^{R}$* | 121,000 | 6,468 | Yes |
| *EHMT$^{+}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 108,000 | 5,773 | Yes |

**Comparisons for Females at 50 % alive-Time Point:**

| Comparison | Diff of Ranks | q | P<0,05 |
|---|---|---|---|
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{+}$; for$^{S2}$* | 489,000 | 8,854 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{+}$; for$^{R}$* | 353,500 | 7,668 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{+}$; for$^{S}$* | 264,500 | 7,155 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 211,500 | 7,597 | Yes |
| *EHMT$^{DD1}$; for$^{s}$* vs *EHMT$^{DD1}$; for$^{R}$* | 142,500 | 7,617 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 346,500 | 7,517 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{R}$* | 211,000 | 5,708 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{s}$* | 122,000 | 4,382 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 69,000 | 3,688 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{S2}$* | 277,500 | 7,506 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{R}$* | 142,000 | 5,101 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{s}$* | 53,000 | 2,833 | Yes |
| *EHMT$^{+}$; for$^{s}$* vs *EHMT$^{+}$; for$^{S2}$* | 224,500 | 8,064 | Yes |
| *EHMT$^{+}$; for$^{s}$* vs *EHMT$^{+}$; for$^{R}$* | 89,000 | 4,757 | Yes |
| *EHMT$^{+}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 135,500 | 7,243 | Yes |

**Comparisons for Females at 80 % alive-Time Point:**

| Comparison | Diff of Ranks | q | P<0,05 |
|---|---|---|---|
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{+}$; $for^{S2}$ | 375,000 | 6,790 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{+}$; $for^{R}$ | 338,500 | 7,343 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{+}$; $for^{s}$ | 256,500 | 6,938 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{DD1}$; $for^{S2}$ | 182,500 | 6,556 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{DD1}$; $for^{R}$ | 146,500 | 7,831 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{+}$; $for^{S2}$ | 228,500 | 4,957 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{+}$; $for^{R}$ | 192,000 | 5,194 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{+}$; $for^{s}$ | 110,000 | 3,951 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{DD1}$; $for^{S2}$ | 36,000 | 1,924 | No |
| $EHMT^{DD1}$; $for^{S2}$ vs $EHMT^{+}$; $for^{S2}$ | 192,500 | 5,207 | Yes |
| $EHMT^{DD1}$; $for^{S2}$ vs $EHMT^{+}$; $for^{R}$ | 156,000 | 5,604 | Yes |
| $EHMT^{DD1}$; $for^{S2}$ vs $EHMT^{+}$; $for^{s}$ | 74,000 | 3,955 | Yes |
| $EHMT^{+}$; $for^{s}$ vs $EHMT^{+}$; $for^{S2}$ | 118,500 | 4,257 | Yes |
| $EHMT^{+}$; $for^{s}$ vs $EHMT^{+}$; $for^{R}$ | 82,000 | 4,383 | Yes |
| $EHMT^{+}$; $for^{R}$ vs $EHMT^{+}$; $for^{S2}$ | 36,500 | 1,951 | No |

**Comparisons for Males at 20 % alive-Time Point:**

| Comparison | Diff of Ranks | q | P<0,05 |
|---|---|---|---|
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{+}$; $for^{S2}$ | 354,500 | 6,419 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{+}$; $for^{s}$ | 309,500 | 6,714 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{+}$; $for^{R}$ | 309,500 | 8,372 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{DD1}$; $for^{S2}$ | 116,000 | 4,167 | Yes |
| $EHMT^{DD1}$; $for^{R}$ vs $EHMT^{DD1}$; $for^{s}$ | 17,500 | 0,935 | No |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{+}$; $for^{S2}$ | 337,000 | 7,311 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{+}$; $for^{s}$ | 292,000 | 7,899 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{+}$; $for^{R}$ | 292,000 | 10,489 | Yes |
| $EHMT^{DD1}$; $for^{s}$ vs $EHMT^{DD1}$; $for^{S2}$ | 98,500 | 5,265 | Yes |
| $EHMT^{DD1}$; $for^{S2}$ vs $EHMT^{+}$; $for^{S2}$ | 238,500 | 6,451 | Yes |
| $EHMT^{DD1}$; $for^{S2}$ vs $EHMT^{+}$; $for^{s}$ | 193,500 | 6,951 | Yes |
| $EHMT^{DD1}$; $for^{S2}$ vs $EHMT^{+}$; $for^{R}$ | 193,500 | 10,343 | Yes |
| $EHMT^{+}$; $for^{R}$ vs $EHMT^{+}$; $for^{S2}$ | 45,000 | 1,616 | No |
| $EHMT^{+}$; $for^{R}$ vs $EHMT^{+}$; $for^{s}$ | 0,000 | 0,000 | No |
| $EHMT^{+}$; $for^{s}$ vs $EHMT^{+}$; $for^{S2}$ | 45,000 | 2,405 | No |

**Comparisons for Males at 50 % alive-Time Point:**

| Comparison | Diff of Ranks | q | P<0,05 |
|---|---|---|---|
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 337,500 | 6,111 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S}$* | 334,500 | 7,256 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{R}$* | 273,000 | 7,385 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 162,500 | 5,837 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{DD1}$; for$^{S}$* | 77,500 | 4,143 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs EHMT(+);s2 | 260,000 | 5,640 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{+}$; for$^{S}$* | 257,000 | 6,952 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{+}$; for$^{R}$* | 195,500 | 7,023 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 85,000 | 4,543 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{S2}$* | 175,000 | 4,734 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{S}$* | 172,000 | 6,178 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$* vs *EHMT$^{+}$; for$^{R}$* | 110,500 | 5,906 | Yes |
| *EHMT$^{+}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 64,500 | 2,317 | No |
| *EHMT$^{+}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S}$* | 61,500 | 3,287 | No |
| *EHMT$^{+}$; for$^{S}$* vs *EHMT$^{+}$; for$^{S2}$* | 3,000 | 0,160 | No |

**Comparisons for Males at 80 % alive-Time Point:**

| Comparison | Diff of Ranks | q | P<0,05 |
|---|---|---|---|
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 320,000 | 5,794 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S}$* | 313,500 | 6,801 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{+}$; for$^{R}$* | 313,500 | 8,480 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 142,500 | 5,119 | Yes |
| *EHMT$^{DD1}$; for$^{R}$* vs *EHMT$^{DD1}$; for$^{S}$* | 113,500 | 6,067 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{+}$; for$^{S2}$* | 206,500 | 4,480 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{+}$; for$^{S}$* | 200,000 | 5,410 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{+}$; for$^{R}$* | 200,000 | 7,184 | Yes |
| *EHMT$^{DD1}$; for$^{S}$* vs *EHMT$^{DD1}$; for$^{S2}$* | 29,000 | 1,550 | No |
| *EHMT$^{DD1}$; for$^{S2}$*vs *EHMT$^{+}$; for$^{S2}$* | 177,500 | 4,801 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$*vs *EHMT$^{+}$; for$^{S}$* | 171,000 | 6,143 | Yes |
| *EHMT$^{DD1}$; for$^{S2}$*vs *EHMT$^{+}$; for$^{R}$* | 171,000 | 9,140 | Yes |
| *EHMT$^{+}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S2}$* | 6,500 | 0,233 | No |
| *EHMT$^{+}$; for$^{R}$* vs *EHMT$^{+}$; for$^{S}$* | 0,000 | 0,000 | No |
| *EHMT$^{+}$; for$^{S}$* vs *EHMT$^{+}$; for$^{S2}$* | 6,500 | 0,347 | No |

# S4 - All Pairwise Multiple Comparison Procedures (Holm-Sidak method) for Total Triglyceride and Quantification

### Comparisons for Fed strains:

| Comparison | Diff of Means | t | P | P<0,05 |
|---|---|---|---|---|
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{DD1}$*; *for$^{R}$* | 0,433 | 11,249 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,379 | 9,843 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{R}$* | 0,330 | 8,582 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,287 | 7,443 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,276 | 7,176 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{DD1}$*; *for$^{R}$* | 0,261 | 6,768 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,184 | 4,776 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{R}$* | 0,173 | 4,482 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{DD1}$*; *for$^{R}$* | 0,158 | 4,101 | <0,001 | Yes |
| *EHMT$^{+}$*; *for$^{S}$* vs *EHMT$^{DD1}$*; *for$^{R}$* | 0,147 | 3,806 | 0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,118 | 3,075 | 0,013 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{DD1}$*; *for$^{S}$* | 0,103 | 2,667 | 0,035 | Yes |
| *EHMT$^{+}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,0924 | 2,400 | 0,053 | No |
| *EHMT$^{+}$*; *for$^{S2}$*vs *EHMT$^{+}$*; *for$^{R}$* | 0,0541 | 1,406 | 0,299 | No |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,0260 | 0,676 | 0,501 | No |

### Comparisons for Food-Deprived strains:

| Comparison | Diff of Means | t | P | P<0,05 |
|---|---|---|---|---|
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,213 | 5,529 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,205 | 5,314 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{R}$* | 0,202 | 5,245 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,174 | 4,509 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,172 | 4,460 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,165 | 4,294 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,163 | 4,245 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{+}$*; *for$^{R}$* | 0,163 | 4,225 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{R}$* | 0,161 | 4,176 | <0,001 | Yes |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{DD1}$*; *for$^{S}$* | 0,0411 | 1,069 | 0,869 | No |
| *EHMT$^{DD1}$*; *for$^{R}$* vs *EHMT$^{DD1}$*; *for$^{S2}$* | 0,0392 | 1,019 | 0,844 | No |
| EHMT(+); BB vs *EHMT$^{+}$*; *for$^{S2}$* | 0,0109 | 0,284 | 0,998 | No |
| *EHMT$^{+}$*; *for$^{S}$* vs *EHMT$^{+}$*; *for$^{S2}$* | 0,00828 | 0,215 | 0,995 | No |
| *EHMT$^{+}$*; *for$^{R}$* vs *EHMT$^{+}$*; *for$^{S}$* | 0,00266 | 0,0692 | 0,997 | No |
| *EHMT$^{DD1}$*; *for$^{S2}$* vs *EHMT$^{DD1}$*; *for$^{S}$* | 0,00190 | 0,0492 | 0,961 | No |