Discussion

The present study demonstrates that caffeine increased mortality of female *Drosophila* without significantly affecting two major physiological mechanisms that can artifactually modulate mortality. In females, the observed increased mortality at 0.008 mg/ml and 0.08 mg/ml was not associated with any statistically significant reductions in fecundity or metabolic rate. This indicates that while caffeine does not negatively impair reproduction or metabolism, there is an underlying negative health effect in females that results in increased mortality.

Females supplemented with the highest dose, 0.8 mg/ml, did not demonstrate any significant change in mortality. We cannot explain this mortality observation; however, caloric restriction can be ruled-out due to the lack of depressed fecundity.

We evaluated the impact of caffeine on the metabolic rate of both sexes and did not observe any significant changes at any dose. While caffeine is a thermogenic stimulant and was presumed to increase the metabolic rate, no such change was observed. This can be attributed to the fact that *Drosophila melanogaster* naturally have a prominently constant metabolic rate that cannot easily be altered (Promislow and Haselkorn, 2002).

We have presented evidence that caffeine can significantly increase mortality in female *Drosophila melanogaster* without impacting reproductive and metabolic mechanisms. Further research is needed to investigate the primary effect of caffeine on female mortality.

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Deficiency screen reveals genomic region required for tumorigenesis and metastasis of *lethal (2) giant larvae* brain tumors.

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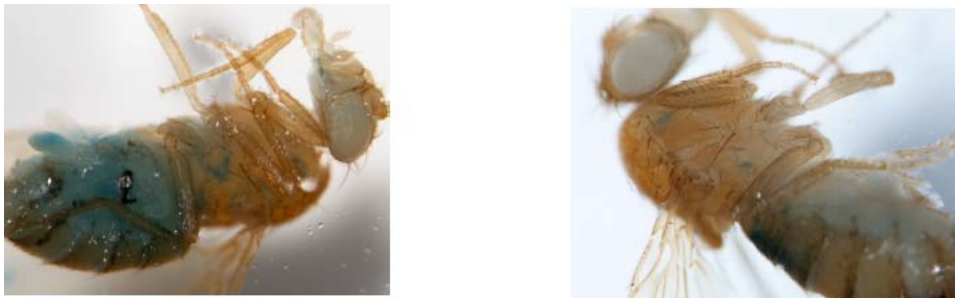
Lack of function mutations in the *lethal giant larvae (lgl)* gene cause neoplasia of the larval brain and imaginal discs (Gateff, 1978). The LGL protein has been shown to be important for the

apical-basal polarity of cells and for regulating the asymmetric division of neuroblasts. *Drosophila* neuroblasts normally give rise to a neuroblast and a terminally differentiating ganglion mother cell (GMC). In *lgl* mutants, with increased frequency, the neuroblasts give rise to two neuroblast daughter cells that continue to divide and result in excessive numbers of neuroblasts (Lee *et al*., 2006). These cells, in turn, can undergo enhanced proliferation, leading to a neoplastic mass. This neoplastic tissue can be transplanted into adult hosts. Upon transplantation, the tissue proliferates and a subset of the cells display migratory and invasive behavior and undergo metastasis (Woodhouse *et al*., 1998). These metastatic cells form secondary tumors in a variety of host sites such as the wings, legs, thorax, head and are capable of invading host structures such as the developing egg chambers of the ovary, gut, eye, and brain.

We undertook a deficiency screen to identify genomic regions that contain genes required for the tumorigenic and metastatic phenotype of *lgl* cells. We have focused on the third chromosome, looking at the tumorigenic and metastatic patterns of brain tumors derived from lines with both a third chromosome deficiency and an *lgl* lack of function mutation.



lgl- *lgl 6457*

Figure 1. 6457 deficiency affects *lethal giant larvae* tumorigenesis and metastasis phenotype.

Materials and Methods

A set of third chromosome deficiencies from the Bloomington Stock Center Deficiency kit were crossed into a heterozygous lgl^4 and homozygous *armadillo-lacZ, yellow*$^-$ background. The lgl^4 chromosome was balanced with a y^+CyO chromosome which allowed lgl^- homozygotes to be selected on the basis of the y^- phenotype. The *armadillo-lacZ* construct is expressed in all cells of the larval brain, providing a means of detection of *lgl* cells after transplantation into adult hosts. Brain tissue from the *lgl* larvae with the *lacZ* marker and the third chromosome deficiency was dissected; brain lobes were cut into halves and injected with a 33 Gauge needle into the ventral abdomens of wild-type hosts. The hosts were cultured for 21 days following injection and stained for *lacZ* activity to reveal the localization of the *lgl* cells in the hosts. The beta-galactosidase activity was determined using a Specialty Media staining kit. The host abdomens were opened along the ventral midline and the hosts were washed once in Tissue Rinse Solution A. The hosts were then incubated in Tissue Rinse Solution A for 30 minutes, followed by a rinse and a 5 minute wash in Tissue Rinse Solution B. The hosts were stained overnight at 37^o in the X-gal stain solution. Hosts were scored for the following criteria: size of the primary tumor, number of egg chambers with invasive cells, number of secondary tumor foci in the thorax, and the presence of secondary tumors in the head, eyes, mouthparts, legs, haltere, and wing.

Results

We screened a panel of third chromosome deficiencies for effects on the metastasis of *lgl* cells. One of the third chromosome deficiency *lgl* double mutant lines, *lgl*^{4} 6457, showed a dramatic alteration in the metastatic patterns compared to *lgl* alone (Table 1). Hosts injected with *lgl*^{4} larval brain fragments developed primary tumors in the abdomen, had invasion of egg chambers, and secondary tumors in the thorax, head, leg, and wings. The percentage of host target regions with secondary tumors, referred to here as a metastatic index, was calculated for *lgl*^{4} and *lgl*^{4} *6457* transplanted tissue. The metastatic index for *lgl* transplanted tumor tissue was 0.32. The 6457

Table 1. Metastasis patterns of *lgl*, *lgl 6457*, *lgl CG11279*, *lgl Caps* and *lgl tartan* mutants

Line	n=	eggs	thorax	head	eye	mouth parts	leg	Haltere/wing	M.I.
lgl4	12	7.2	4.7	4/12=33%	7/12=58%	5/12=42%	3/12=25%	0/12	0.32
lgl4 6457	33	2.4	2.4	2/33=6%	5/33=15%	4/33=12%	3/33=9%	1/33=3%	0.09
lgl4 CG11279	14	2.9	4.6	4/14=29%	4/14=29%	4/14=29%	7/14=50%	2/14=14%	0.3
lgl4 CapsEP3557	10	12.2	6.5	5/10=50%	6/10=60%	5/10=50%	3/10=30%	2/10=20%	0.42
lgl4 tartanS064117	10	9.7	5.6	2/10=20%	6/10=60%	5/10=50%	2/10=20%	1/10=10%	0.32

Table 2. Genes Mapped Chromosome 3L 69F6-70A2

Cytology	Sequence Coordinates	Symbol	Protein function
69F6	3L:13022064..13029537	CG17672	249 aa, no putative homology regions
69F6	3L:13029926..13034076	SRm160	structural constituent of ribosome
69F6	3L:13034856..13037012	RpS4	structural constituent of ribosome
69F6	3L:13035867..13036006	snoRNA:Psi28S-3327a	Small nucleolar RNAs
69F6	3L:13036044..13036183	snoRNA:Psi28S-3327b	Small nucleolar RNAs
69F6	3L:13036471..13036610	snoRNA:Psi28S-3327c	Small nucleolar RNAs
69F6	3L:13037536..13040001	Syx13	protein transporter activity t-SNARE family
69F6	3L:13040124..13040623	CG11279	102 aa, no conserved domains
69F6	3L:13053622..13054494	CG14115	219 aa, no conserved domains
69F6	3L:13054979..13055787	CG34428	230 aa, no conserved domains
69F6	3L13056146..13057029	CG34429	249 aa, no conserved domains
70A1	3L:13079487..13080389	CG17300	hydrogen-transporting ATP synthase activity
70A1	3L:13081912..13082928	CG11251	carboxy-lyase activity, isomerasae activity
70A1	3L:13091351..13091728	CG32118	125 aa, no putative homology regions
70A1	3L:13107373..13111188	trn	Involved in: cell adhesion; open tracheal system development; cell migration; apoptosis; signaling
70A1	3L:13129369..13129839	CG33262	137 aa, no putative homology regions
70A2	3L:13166782..13169797	snky	Involved in: fertilization, exchange of chromosomal proteins; sperm chromatin decondensation; sperm plasma membrane disassembly
70A2	3L:13204314..13204385	CR32129	tRNA_gene
70A2	3L:13204651..13204722	CR32128	tRNA_gene
70A2	3L:13205465..13205536	CR32126	tRNA_gene
70A2	3L:13207209..13207333	CR32127	tRNA_gene

deficiency combined with *lgl* resulted in a much lower metastatic index, 0.09. All host regions assayed showed a lower frequency of metastasis. The average number of egg chambers per host that were invaded by *lgl* as compared to *lgl 6457* cells dropped from 7.2 to 2.4. The number of secondary tumors in the thorax was also lower (4.7 for *lgl* as compared to 2.4 for *lgl 6457*, on average). All other metastasis target sites scored lower in the presence of the 6457 deficiency. For example, the frequency of secondary tumors in the head dropped from 33% for *lgl* mutant tissue to 6% for *lgl 6457* mutant tissue. Significantly, eye metastasis, which includes a clear invasive component, dropped from 58% in *lgl* mutants to 12% in *lgl 6457* (Table 2). Overall, the reduction in metastatic index from 0.32 for *lgl* to 0.09 for *lgl 6457* was a reduction of over 70% in the *lgl* metastatic index.

The 6457 deficiency spans the genomic region 3L 69F6-70A2, a region of the third chromosome containing 21 genes. The genes in this region are listed in Table 2. We have tested some of the genes in the region directly for effects on *lgl* metastasis. Double mutants of *lgl* and the *tartan* gene were generated for analysis of metastasis patterns. *tartan* was an attractive candidate, considering its role in motility and cell guidance in *Drosophila* tracheal development (Kause *et al*., 2006). Analysis of the metastasis patterns, however, showed no change in the metastatic index of *lgl tartan* compared to the *lgl* mutation alone. The *capricious* gene was also tested due to the high degree of functional similarity with *tartan* and the proximity of *capricious* to the 3L 69F6-70A2 region. In fact, a capricious mutant was used to generate the 6457 deficiency (Parks *et al*., 2004). The metastasis of *lgl capricious* double mutant issue was not reduced compared to *lgl* mutant tissue (0.42 compared to 0.32) (Table 1). We also tested the *CG11279* gene as a candidate for the tumorigenesis/metastasis suppressor within the 3L 69F6-70A2 region, as a P element mutation that disrupted this gene was available from the Bloomington Stock Center. We observed no significant difference in the metastatic index of the *lgl CG11279* mutants compared to *lgl* (0.3 compared to 0.32) (Table 1). In summary, we have identified the 3L 69F6-70A2 genomic region affected in the deficiency line 6457 as containing a gene or genes important for *lgl* tumorigenesis and metastasis. We have tested a set of candidate genes in the 3L 69F6-70A2 region and have ruled out these genes as the responsible gene for the inhibition of *lgl* tumorigenesis and metastasis we observed for the 6457 deficiency. Further study should identify this gene. Mutations in a number of the genes in the 3L69F6-70A2 region are available exclusively from the Vienna Drosophila RNAi Center (RpS4, CG11251, CG32118, CG33262, and snky). Other mutations are also available from the Bloomington Stock Center or the Exelixis Stock Center at Harvard Medical School, namely CG17672, Syx13, and CG14115. The possibility exists that more than one gene must be deleted to observe the tumorigenesis/metastasis inhibition phenotype. In this case, a similar analysis of smaller deletions in the area in combination with the *lgl* mutation may be useful.

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