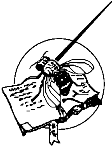

D. melanogaster and *H. irritans* are more sensitive to Phloxine B phototoxicity than *C. capitata.*

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The use of photosensitizers is emerging as an ambient friendly possible new tool to control several insect species. Xanthene derivatives have been the most extensively studied (Heitz, 1997; Ben Amor and Jori, 2000, for review). The toxicity of these dyes has been tested in the adult stage of different insect orders (ants: David and Heitz, 1978; boll weevil: Callaham *et al.*, 1975; cockroaches: Ballard *et al.*, 1988; grasshoppers: Capinera and Squitier, 2000). In particular, xanthenes showed acute photo-toxicity against several Dipterans (*Musca domestica*: Pimprikar *et al.*, 1980; *Culex* and *Aedes*: Pimprikar *et al.*, 1979; *Ceratitis capitata*: Liquido *et al.*, 1995; *Anastrepha ludens*: Mangan and Moreno, 1995). Studies of the effects in the dark as well as in immature stages must be carried out in order to understand which phototoxic pathway is triggered and which main cellular and sub-cellular targets are affected. However, the effects of these dyes against immature stages of dipterans have been seldom analyzed. Among the few studies, Pimprikar *et al.* (1980) studied Erythrosin B effects in *Musca domestica*, whereas Berni *et al.* (2003) studied the toxicity of Phloxine B in *Ceratitis capitata*.

In a preliminary approach, the aim of this work was to compare the toxic effect of Phloxine B (PhB) during the postembryonic development of *D. melanogaster* (*D.m.*), *Haematobia irritans* (*H.i.*), and *Ceratitis capitata* (*C.c.*) and to determine which stage was first affected.

The different larval media with or without Phloxine B disodium salt (D&C Red Nº 28, Warner Jenkinson, St Louis, MO) were prepared just before the experiments. Each experiment was carried out in not less than 8 replications. Batches of 30 newly hatched larvae I of *D. melanogaster* (strain Oregon R) were placed on the surface of Formula 4.24 Instant *Drosophila* Medium (Carolina Biological Supply). *C. capitata* wild type (strain "Arg-17") were reared in a novel pumpkin-based medium (100 g of processed crude organic pumpkin, 100 g cooked pumpkin, 100 g corn flour, 20 g powdered yeast, 50 g sucrose, 0.7 g sodium benzoate, 1.0 g Nipagin M and peach juice in a final volume of 500 ml and adjusting a pH of 4.5 with 10% HCl). These batches of 75 larvae I were placed on small circles of filter paper (Whatman 1) located on the surface of the food. Both cultures of *D.m.* and *C.c.* larvae were maintained in a Conviron chamber CMP 3244, at 23ºC, 50-60% RH. The synchronization attained in cultures of *C.c.* is usually within 65-75% accuracy, whereas synchronization in *D.m.* is less accurate. Batches of 20 newly hatched larvae I of wild type *H. irritans* were seeded on the surface of a cotton gauze embedded with bovine feces extract with or without Phloxine B. The cultures were placed in a culture chamber and maintained, at 30 ºC and 100% humidity. Two hours before puparium formation (BPF), larvae III were moved from the chamber, separated from the cotton gauze and placed in a plastic Petri plate.

Figure 1 A shows schematically that all the flies were maintained in the dark during the larval stages and exposed to light (5000 lux) during the wandering (or equivalent) average period (WAP). After pupariation, the pharate stages were maintained in the dark until imago ecdysis (Figure 1 A).

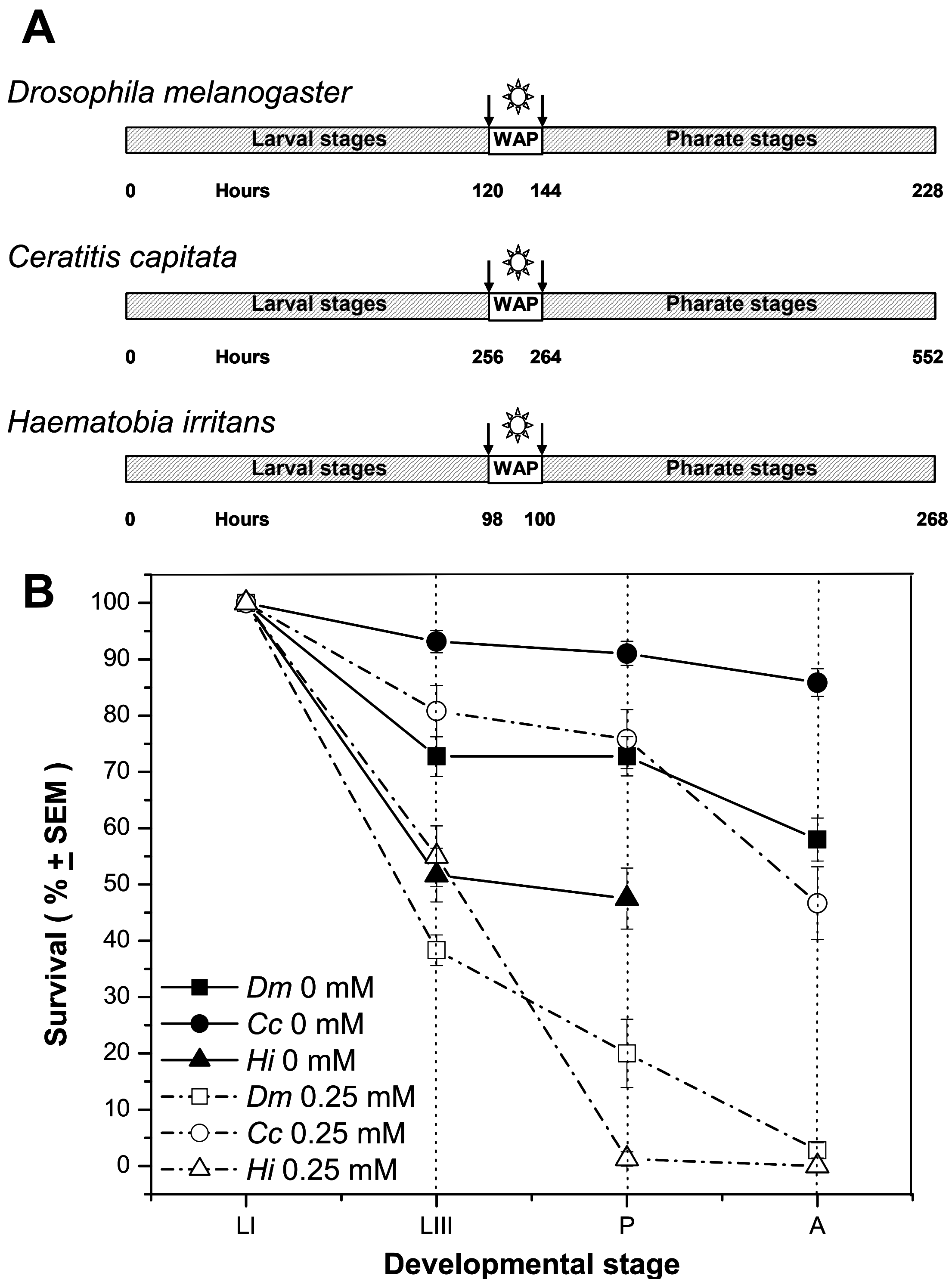

Figure 1. (A) Experimental design employed in this study. Each fly was exposed to light (5000 lux) during the wandering average period (WAP). Hours indicate the span of larval stages, wandering period and pharate stages for each fly. LI indicates larvae I, LIII indicates larvae III, P indicates pupariation and A indicates imago ecdysis. (B) Percentage of survival during the postembryonic development of *Drosophila melanogaster* (■, □), *Ceratitis capitata* (●, ○) and *Haematobia irritans* (▲, Δ) reared with or without Phloxine B. Solid symbols indicate larvae reared without PhB in the

(Figure 1 legend, continued) larval medium (control); indicate larvae reared with PhB 0.25 mM in the larval medium. Each point in the figure represents the average of 8 replications.

The protocols were adapted to each life cycle; the span in hours of the normalized and synchronized flies is shown in Figure 1 A. We determined that *D.m.* 50% lethal concentration (LC_{50}) for PhB was 0.06 mM, whereas for *C.c.* was 0.46 mM, thus showing that *D.m.* exhibits higher overall sensitivity. We selected an intermediate concentration of PhB to compare the effect in the three studied flies.

Figure 1 B shows the comparative survival data of the three flies when 0.25 mM PhB was included in the food. We know from previous experiments that probably less than 1% of the ingested food reaches the internal organs, since most of the dye is retained by the gut cells. The percentage of survival during postembryonic development (from LI to adult ecdysis) with light exposure during the wandering period differs in each of the flies analyzed. The percentage of *C.c.* survival at adult ecdysis was 46.68% ± 6.46 (with 0.25 mM PhB), whereas control without PhB was 85.87% ± 2.46. *D.m.* and *H.i.* were more sensitive to PhB treatment, showing a percentage of survival of 2.77% ± 1.33 and 1.25% ± 1.25, respectively. When *D.m.* and *C.c.* were maintained in complete darkness during postembryonic development, PhB showed practically no effect (*D.m.*: 67.08% ± 3.81 without PhB and 63.04% ± 5.85 with 0.25 mM PhB; *C.c.*: 91.90% ± 5.03 and 83.22% ± 6.51, respectively).

The sensitivity of flies to PhB during the different developmental stages is shown in Figure 1 B. *D.m.* larval stages subjected to 0.25 mM PhB in the food, showed a survival of 38.33% ± 2.72, whereas without the dye the survival rate was 72.77% ± 8.68. *C.c.* was less affected by PhB during larval stages, whereas the survival of *H.i.* seemed not affected. Figure 1 B shows that exposure to light during the wandering period was lethal to the remaining 0.25 mM PhB-treated *H.i.*, since practically no larvae reached pupariation (1.25% of survival). On the other hand, no mortality was recorded in the wandering *H.i.* larvae from controls. No additional mortality was registered during the *C.c.* "jumping" period (equivalent to wandering period in other flies); but, some phototoxicity-related additional deaths were recorded during the pharate stages. No additional *D.m.* deaths were registered during pharate stages.

The above data indicate that, after exposure to light, the fly that appeared to be most sensitive to Phloxine B photoactivation was *Haematobia*; whereas in the same conditions, less mortality was recorded in *Drosophila* and in *Ceratitis*. Unexpectedly, larval stages of *Drosophila* were the most sensitive to Phloxine B in the dark, whereas *Haematobia* larvae were insensitive.

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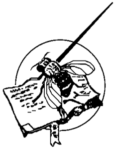

Different response to infection in *Drosophila melanogaster*, *Ceratitis capitata*, and *Tenebrio molitor*.

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Insects trigger a multifaceted innate immune response to fight microbial infections. The first line of defense is the physical cuticle barrier, including the lining of the gut and the perithrophic membrane. When the cuticle is wounded and the insect is infected by a pathogen, three main strategies of defense are displayed, to avoid the propagation of infection (Hoffmann and Reichardt, 2002; Hoffmann, 2003; Naitza and Ligoxygakis, 2004). The first line of response to microbial entry is mediated by the rapid action of phenoloxidases. Phenol oxidoreductases generate quinones that lead to localized melanization and cross-linking (Ashida and Bray, 1995); eventually forming melanotic structures that encapsulate invaders (Ashida and Bray, 1995; Nappi and Ottaviani, 2000). There are also poorly understood mechanisms of hemolymph clotting (Nappi and Ottaviani, 2000), and complement-like response (Lagueux *et al*., 2000). These humoral events are followed immediately by a cellular defense mechanism mediated by hemocytes (Hoffmann and Reichardt, 2002; Hoffmann, 2003; Naitza and Ligoxygakis, 2004; Ferrandon *et al*., 2004; Agaisse *et al*., 2003; Tzou *et al*., 2002). These cells interact with foreign pathogens or materials, displaying a phagocytic behavior that clears invaders from the hemocoel. The third line of defense involves the induction of anti-microbial peptides and proteins that are synthesized by both epidermis and fat body (Hoffmann and Reichardt, 2002; Hoffmann, 2003; Naitza and Ligoxygakis, 2004; Ashida and Bray, 1995). These mechanisms are activated as soon as pathogens, parasitic arthropods, or predators disrupt the integument (local response) and antigens enter into the hemocoel (systemic response).

Hopkins *et al*. (1998) reported that catecholamine biosynthesis seems transiently stimulated in the case of parasitized tobacco hornworm (*Manduca sexta)* larvae. These authors showed that, unexpectedly, N-β-alanyldopamine (NBAD), which is the main sclerotization precursor of insect brown cuticle, was induced by the parasitoid. Moreover, Kim *et al*. (2000) observed an increase of NBAD in the yellow mealworm beetle *Tenebrio molitor* infected with bacteria. The synthesis of NBAD is periodically induced in epidermis, but only at the time of molting (Kramer and Hopkins, 1987), to generate reactive quinones that will cross-link cuticle proteins and chitin chains (Hopkins and Kramer, 1992).

We previously were able to measure, for the first time, the activity of NBAD synthase in the epidermis of the Medfly, *Ceratitis capitata* (Wappner *et al*., 1996). This enzyme conjugates dopamine with β-alanine, and *in vitro* shows a broad substrate activity. It is induced by 20-OH-ecdysone at the time of molting but, as expected, is not active during intermolt periods (Hopkins and Kramer, 1992, Pérez *et al*., 2002). In contrast, we recently demonstrated that, surprisingly, NBAD also is present in neural tissue, but in a constitutive manner (Pérez *et al*., 2004). The enzymatic protein, coded by the *C. capitata* gene *niger* (Wappner *et al*., 1996), is a catalytic homolog of the product of the *Drosophila melanogaster* gene *ebony* (Pérez, 2004).

In order to explore the possibility of NBAD synthase induction due to bacterial challenge, we used *D. melanogaster* and *C. capitata* adults as well as *T. molitor* larvae V. These were injected through the abdominal arthrodial membranes with insect Ringer´solution containing [^{14}C]β-alanine

(30.000 cpm, 250 pmoles) with or without resuspended *Escherichia coli* DH 5α. Volumes of injection were 10 µl for *T. molitor,* 1 µl for *C. capitata*, and 0.2 µl for *D. melanogaster*. The estimated bacteria concentrations (from cultures in logarithmic phase $10.^7$ *E. coli*/ml) were 20,000 *E. coli* in *T. molitor*, 1600 *E. coli* in *C. capitata*, and 300-500 *E. coli* in *D. melanogaster*. If any reflux of hemolymph was detected, the insect was discarded. The injected animals were maintained at 23°C (*T. molitor* and *C. capitata* during 20 h and *D. melanogaster* during 4, 8 or 16h) and then homogenized under liquid N_2. After centrifugation, the soluble material was quantified and analyzed using HPLC (mobile phase: 1 mM sodium octyl sulfate, 2.5 mM potassium chloride, 1 mM Na_2EDTA, and 6% acetonitrile adjusted to pH 2.2 with phosphoric acid).

Figure 1 shows an induction experiment in *T. molitor* larvae. In the absence of bacteria, no NBAD was synthesized (Figure 1, upper pane), whereas infected insects contained [^{14}C]-labeled substances that behaved as NBAD (Figure 1, lower pane).

Induction of NBAD synthase was tested in *T. molitor* larvae, as well as in *C. capitata* and *D. melanogaster* adults by injection of live *E. coli* or 0.1 mg/ml *E. coli* lipopolysaccharides (LPS) in *T. molitor* and *D. melanogaster*. After separate homogenization of bodies and heads, cell-free synthase activities were measured as previously described (Pérez *et al*., 2002). The *in vitro* synthesized radiolabeled substances bound to alumina, therefore behaving as catecholamines, were analyzed in HPLC and counted in a scintillator counter. Table 1 shows that as expected, the heads of *D. melanogaster* and *C. capitata* exhibited significant NBAD synthase activity, which was not enhanced when *Escherichia coli* was injected. When the carcasses of *C. capitata* were analyzed, we found that a strong induction of NBAD synthase occurred when *E. coli* was injected, while negligible activity was detected in controls (Table 1). However, injection of *E. coli* or *E. coli* LPS in *D. melanogaster* was unable to trigger the induction of NBAD synthase activity (Table 1). Therefore, this peculiar NBAD synthase induction, presumably associated to the innate immune response, seems to be absent in *D. melanogaster*. However, induction in *T. molitor* does occur (Figure 1 and Table 1), thus indicating that probably this is a response widely present in insects. Experiments are under way to determine if a different isoform of *ebony* coded NBAD synthase, arboring a different substrate specificity, might be expressed in *Drosophila* challenged with bacteria.

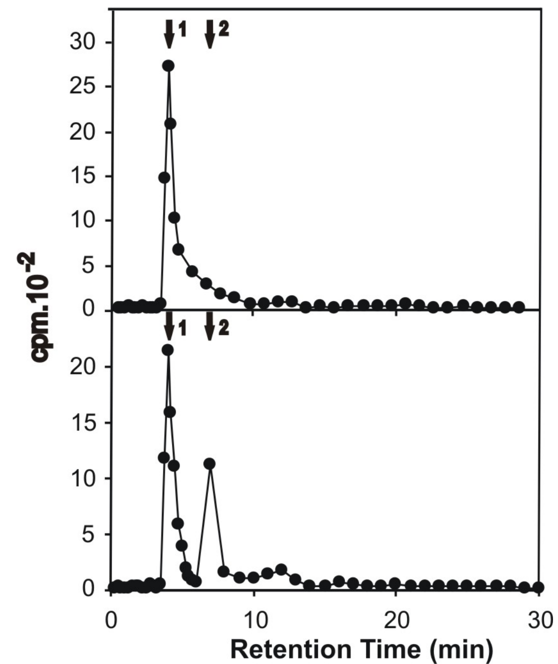

Figure 1. HPLC analysis of in vivo induction of NBAD synthesis. Non-sclerotizing T. molitor larvae V were injected with a micro-needle. Injections of 13 µl insect ringer contained [^{14}C]β-alanine as substrate tracer without bacteria (upper panel) or with bacteria (lower panel). Arrows indicate the retention time of internal standards of β-alanine (1) and NBAD (2).

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Table 1. *In vitro* NBAD synthase activity induction by live *E. coli* or *E. coli* lypopolysaccharide.

Insect	control	*E. coli*	LPS
D. melanogaster[1]			
Head	43.00 ± 7.00	42.00 ± 6.81	–
Body	0.74 ± 0.29	1.22 ± 0.64	1.06 ± 0.41
C. capitata[2]			
Head	9.06 ± 1.37	10.03 ± 2.85	–
Body	0.51 ± 0.71	12.52 ± 3.58	–
T. molitor[3]	0.72 ± 0.17	29.79 ±15.45	60.97 ± 15.45

1: *Drosophila melanogaster* 5 days old adults.
2: *Ceratitis capitata* 7 days old adults.
3: *Tenebrio molitor* intermolt larva V.0
Experiments were carried out at least in triplicate.

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Pifithrin-β potentiates somatic mutagenesis and tumor growth in *D. melanogaster*.

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Recent studies identified chemical inhibitors of the function of p53 protein in mammalian cells and organisms, pifithrins α and β (Komarova and Gudkov, 2000; Brannon-Peppas *et al*., 2004). Both chemicals mimic the effects of *p53* mutation in mammals preventing the *p53* transactivation effects such as mutagen-induced cell death (Komarova *et al*., 2003). The discoverers hypothesize that pifithrin affects the nuclear transport of p53 (Komarova and Gudkov, 1999). It was shown that at 37°C pifithrin-α undergoes a spontaneous conversion into pifithrin-β; the same effect takes place *in situ*. (Approximately one-half of pifithrin-α turns to pifithrin-β in 3 hrs.) (Brannon-Peppas *et al*., 2004).

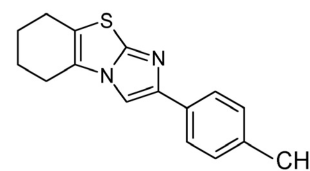

Figure 1. Structural formula of pifithrin-β.

We have studied the mutagenic and blastomogenic properties of pifithrin-β (Figure 1) in heterozygotes for *wts* tumor suppressor gene capable to develop benign tumorous mosaic clones in response to a chemical treatment. Pifithrin-β was applied solo, as a 0.05 mg/ml suspension in 10% aqueous dimethylsulfoxide (DMSO) solution,

and in the same concentration in combination with 0.2 mg/ml oxoplatin (diamminedichlorodihydroxyplatinum IV, a super-mutagenic anti-cancer drug), also diluted in 10% DMSO. As a control, the following variants of treatment were used: 0.2 mg/ml water solution of oxoplatin, 10% DMSO and distilled water. Differences of tumor frequency induced by distilled water and 10% DMSO solution were found statistically insignificant. For comparison, we also measured the effect of 0.2 mg/ml oxoplatin in *wts*/+ heterozygotes bearing a dominant-negative mutation in the *p53* gene ($p53^{259H.GUS}$) with distilled water as a control.

All the chemicals were applied to the first instar, 36 hrs-old larvae, a progeny of the cross: females *w; P{*w^{+mC}*}*wts^{P2} */ TM6B, Hu Tb e* × D-32 (wild type), or *w*; *P{*w^{+mC}=$p53^{259H}$*}* males. Adults (only *wts*/+ heterozygotes without markers of the balancer) were examined under a stereomicroscope for tumor clones. Clone frequency was calculated as p = (number_of_tumos)/(number_of_flies) × 100% and compared to control frequencies in a Student t-test with Fisher's correction.

The results are summarized in the Figure 2. We have found that β-pifithrin itself is a weak mutagen with the activity slightly exceeding the control values ($P < 0.05$). This level of mosaicism is comparable to that induced by benzo(e)pyrene (data not shown). However, together with a strong mutagen, oxoplatin, pifithrin-β dramatically (~ 6 times, from ~ 16% to ~ 97%) potentiates its influence on the tumor formation. The effect of the combined treatment by oxoplatin and pifithrin is very comparable to the enhancement, which a dominant-negative mutation $p53^{259H}$ exerts on the activity of oxoplatin (it also raises the oxoplatin-induced tumor frequency ~ 6 times, up to ~ 100%).

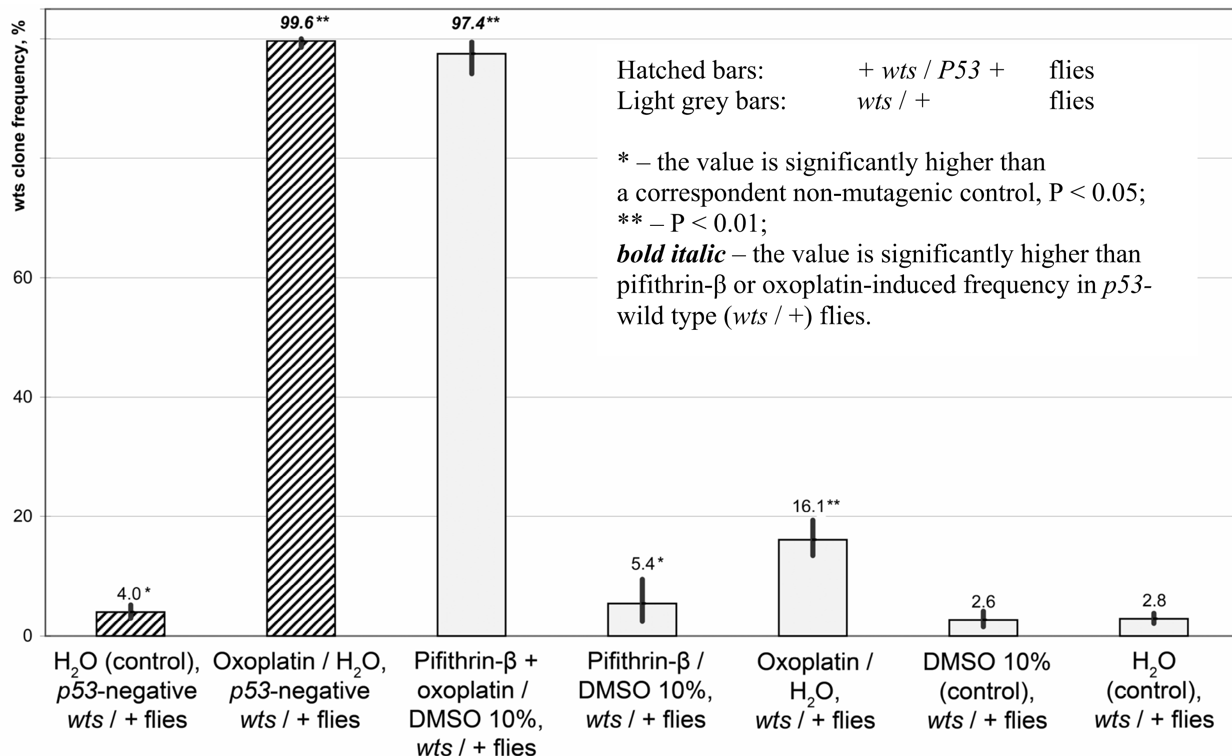

Figure 2. Tumor frequencies induced by pifithrin-β, oxoplatin and their mixture in *p53*-negative and *p53*-positive heterozygotes for the tumor suppressor *wts*.

So, in *Drosophila*, pifithrin-β acts as a weak mutagen, but a powerful enhancer of a mutagenic effect of other substances. This may be related to its negative effects on the mutagen-induced apoptosis of cells with pre-mutagenic lesions. On the other hand, our results allow us to speculate that the degree of similarity of the p53 proteins and *p53* pathways in mammals (mice and human) and *Drosophila* is enough to allow pifithrin to act in the same manner.

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Genetic correlation between types of mtDNA of *Drosophila melanogaster* and genotypes of its primary endosymbiont, *Wolbachia.*

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Bacteria of *Wolbachia* genus belong to the group of alpha-proteobacteriae of Rickettsia class, being the primary endosymbionts of a wide range of Arthropoda and filarial-nematode species. The spread of *Wolbachia* in the host species population is thought to be associated with induction of reproductive abnormalities, such as cytoplasmic incompatibility, parthenogenesis, feminization, and androcide.

Wolbachia are classified based on their nucleotide sequence; however, within *Drosophila melanogaster* as a host they are monomorphic by this criterion. Five polymorphic markers are used for genotyping of *Wolbachia* infecting *Drosophila melanogaster*, as follows: insertion into two loci of *IS5* insertion sequence, minisatellites or variable number tandem repeats (VNTRs) and the inversion. We analyzed a single imago fly from isofemale line, each of those representing the progeny of a female fertilized in the wild and maintained henceforth in the collection of the Laboratory of Genetics of Populations, Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences. Screening of laboratory stocks and lines obtained from the geographically distant *Drosophila melanogaster* populations throughout the world revealed 5 *Wolbachia* genotypes. Two of them, wMel и wMelCS, showed worldwide occurrence, while wMelCS2 was found in Eastern Europe and Central Asia only, and wMel2, found solely in Japan. wMel3 found in a single fly stock was, presumably, generated in the course of lab cultivation (Riegler *et al*., 2005).

There is a correlation between the *Wolbachia* infection of the host species and the descent of mitochondrial DNA. As long as the transmission of *Wolbachia* and mitochondria in the generations of host species occurs through the cytoplasm, there is an indirect effect of bacteria on the diversity, distribution, and fixation of the new variants of mitochondrial mitotypes (DeWayne *et al*., 2004; Ballard, Kreitman, 1994).

In those host species for which different strains of *Wolbachia* are known, a more thorough description of the cytoplasmic genomic components is possible, particularly, identification of

cytotypes including the characteristic of an individual (or strain) by the mitotype of the host mtDNA and the infection status of a bacterial strain/genotype. Persistence of particular cytotypes was demonstrated for a number of species: *Culex pipiens* mosquitos (Vinogradova *et al*., 2003; Shaikevich, 2005), *Solenopsis invicta* ants (Shoemaker *et al*., 2003), and some *Drosophila* species of *melanogaster* group (Solignac, 2004). However, the correlation between *Wolbachia* infection and the host mtDNA mitotype have not yet been demonstrated for *Drosophila melanogaster* (Solignac *et al*., 1994).

Lack of evident correlation between *Wolbachia* infection and mtDNA mitotype in *Drosophila melanogaster* can be, first, accounted for by monomorphism of *Wolbachia* for *wsp* gene, and second, by the lack or weak expression of cytoplasmic incompatibility, a reproductive abnormality induced by *Wolbachia.*

Identification of cytotypes for *Drosophila melanogaster* as a model organism is an important approach to the understanding and simulation of the processes of inheritance of the cytoplasmic genomic components in the population, the possibility of horizontal transfer, and the reconstruction of species history.

Table 1. Correlation between *Wolbachia* infection and the co1 gene mitotypic diversity in *Drosophila melanogaster*.

Mitotype of *D. melanogaster*	Number of non-infected lines	Number of infected lines with the known infection status and *Wolbachia* genotype		
		wMel	wMelCS2	wMelCS
CT	14	15	0	0
CC	1	0	4	0
TC	2	0	0	5

In our work we first found the correlation between mitochondrial mitotypes of the first subunit cytochrome oxidase C gene of *Drosophila melanogaster* and *Wolbachia* genotypes. We determined the 499 bp mtDNA sequence of first cytochrome oxidase C subunit gene from region 2151-2649 (according to the annotated complete mtDNA genome sequence of Oregon R stock - AN: AF200828) from infected and non-infected isofemale lines of *Drosophila melanogaster* differing in *Wolbachia* genotypes. In the studied lines isolated from the natural populations of *Drosophila melanogaster*, we found two polymorphic sites of the mitochondrial genome of *Drosophila melanogaster* differing from the annotated complete mtDNA genome sequence of Oregon R stock - AN: AF200828, nucleotide substitution positions 2160 (C/T) and 2187 (C/T). For 29 out of 41 sequenced isofemale lines of *Drosophila melanogaster* we established CT mitotype (2160 C, 2187 T), which is similar in the studied region to the annotated complete mtDNA genome sequence AF200829 (Zimbabwe 52) and AJ400907; 7 lines showed TC mitotype similar to AF200828 (Oregon R); while 5 lines demonstrated a newly described CC mitotype (Table 1).

The comparison of infection status and the genotyping of the infected isofemale lines of *Drosophila melanogaster* revealed that all of the infected wMel-*Wolbachia* lines as well as 14 out of 17 non-infected lines were defined as CT mitotype. TC mitotype was found in 5 lines infected by *Wolbachia*-wMelCS genotype, as well as in 2 non-infected lines.

The prevalence of the newly described CC mitotype correlates with wMelCS2 *Wolbachia* genotype: 4 infected and a non-infected line had this mitotype.

Thus, we establish the evolutionary link between the mitochondrial inheritance in *Drosophila melanogaster* and the genotypes of the maternally transferred primary endosymbiont, *Wolbachia*.

Acknowledgments: This work was supported by the Russian Foundation for Basic Research, project 05-04-48838, and the Program of the Russian Academy of Sciences "Biodiversity and Dynamics of Gene Pools".

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Wolbachia* in populations of *Drosophila melanogaster.

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An extraordinary evolutionary success of a bacterial symbiont of *Wolbachia* species prevalent among arthropods and nematodes attracts a rapt attention of scholars worldwide. According to different estimates, from 17% to 76% of insect species may be infected by *Wolbachia* (Werren *et al*., 1995; Jeyaprakash and Hoy, 2000). Bacteria are transmitted maternally through the egg cytoplasm to progeny throughout host generations. For a more efficient spread of bacteria in the population, it manipulates the host reproductive system, thence somehow improving the reproductive success of infected individuals.

Drosophila melanogaster could be the most useful model for the study of key aspects of Arthropoda-*Wolbachia* co-existence, from molecular mechanisms to evolutionary patterns and transformations. However, the scholars encountered a number of difficulties and particularities while working with this system.

First, the flies collected directly in the wild, in most cases, cytoplasmic incompatibility is not expressed or observed (Werren, 1997; Mercot and Charlat, 2004; Hoffman *et al*., 1998). It is, however, detected at a low level in the laboratory lines, as a rule, when young males are used for experimentation. This raises the question: how the bacteria spreads over and maintains in the population? Obviously, under particular conditions, infection by *Wolbachia* yields higher fitness for *Drosophila melanogaster*.

Second, the molecular data based on analysis of *16S rRNA* and *ftsZ* genes, while testifying to the similar descent of the bacteria, still cannot help in establishing the phylogeny of bacterial species based on those sequences, as well as a further analysis of a more variable surface protein gene *wsp* was of no more help (Zhou *et al*., 1998).

Analysis of a complete genome of *Wolbachia* strain wMel of *Drosophila melanogaster* set a new milestone in the model study of *Drosophila melanogaster – Wolbachia* association, while revealing a number of particular features of this endosymbiont`s genome: multiple mobile elements, repetitive sequences, and genes coding for proteins containing ankyrin domains, which, obviously, play an important role in the origin of reproductive abnormalities (Wu *et al.,* 2004). The use of loci containing mobile element insertions, repetitive sequences, and chromosome rearrangement regions as markers, offered an opportunity for *Wolbachia* genotyping. Five genotypes were described, differing in the insertions in two loci of *IS5* transposon, different repetitive factor of the two minisatellites, and a long inversion (Riegler *et al*., 2005).

In our work, we studied the occurrence of cytoplasmic bacteria *Wolbachia* in the populations of *Drosophila melanogaster* of the former USSR: Ukraine, Belarus, Moldavia, the Caucasus, Central Asia, Eastern and Western Siberia, and Altai.

Table 1. Occurrence of *Wolbachia* genotypes in the wild populations of *Drosophila melanogaster* from the three regions of Eurasia.

Region	Number of non-infected lines	Number of *Wolbachia*-infected lines of genotype			Lines studied (total)
		wMelCS	*wMelCS2*	*wMel*	
Ukraine	150	0	1	80	231
Central Asia	53	1	17	76	147
Altai, Russia	69	5	19	63	156

The most representative collections came from Ukraine, Central Asia and Altai (Table 1). We detected *Wolbachia*–positive cytoplasm by PCR amplification of *wsp* bacterial genes in the females of isofemale lines maintained in the Collection of the Laboratory of Genetics of Populations of the Institute of Cytology and Genetics as well as the females caught directly in the wild populations of *Drosophila melanogaster*. Where *Wolbachia* infection was detected, bacteria were genotyped according to the five above markers.

As many as 665 isofemale lines obtained from *Drosophila melanogaster* populations of Eurasia – Ukraine, Belarus, Moldavia, the Caucasus, Central Asia, the Urals, Altai, Eastern and Western Siberia, and the Russian Far East over the period of 1974-2005 were used in this study. Most representative were the collections and individual lines deriving from Ukraine (33%), Altai (24%), and Central Asia (24%). The prevalence of infected flies in the populations varied from 15% to 100%. On average, about 39% of flies were infected in the populations of Eastern Europe, 56% in Altai populations, and 64% in Central Asian populations.

Regional differences are also evident in the *Wolbachia* genotypes represented in those regions. We were able to detect three *Wolbachia* genotypes, namely, wMel, wMelCS, and wMelCS2. Genotype wMel is the most ubiquitous and often found, which agrees with the known data for *Drosophila melanogaster* populations throughout the world. Genotype wMelCS2 was once encountered in the populations of Ukraine, as well as in Moldavia and Belarus, while wMelCS genotype was not found there at all.

Heterogeneity of cytoplasmic inheritance factor, *Wolbachia* bacteria, we observed in the populations of *Drosophila melanogaster* of Central Asia and Altai. Here, all three of *Wolbachia* genotypes are found, wMel, wMelCS2, and wMelCS.

The particular features of occurrence of endosymbiont types in populations of *Drosophila melanogaster*, position Central Asian and Altai populations closer to each other than to the Ukrainian collections. This can be attributed to geographic proximity and similarity of natural and climatic conditions of those regions.

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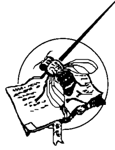
CG32130, *starvin*, is expressed as a heat shock gene in *Drosophila melanogaster*.



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Abstract

A microarray study of genes expressed in thermotolerant and phenocopy sensitive *Drosophila melanogaster* pupae revealed that the BAG domain encoding gene, CG32130, recently named *starvin*, is expressed in a similar manner to the heat shock genes. This observation has been confirmed using real time PCR. Since the BAG genes in mammals encode proteins that are anti-apoptotic and bind Hsp70, this suggests that one aspect of thermotolerance in *Drosophila* may be the prevention of apoptosis by Hsp70 in cooperation with CG32130.

Introduction

Abruptly raising the temperature to 40°C causes death and developmental defects in *Drosophila* larvae, pupae, adults, and cell lines. A thermotolerance inducing treatment of 35°C immediately before the high temperature heat shock can prevent death and developmental defects. During the 35°C treatment, heat shock proteins are synthesized and play a role in the acquisition of thermotolerance (Mitchell *et al.*, 1979; Petersen and Mitchell, 1981). Since heat shock proteins have been shown to be chaperones that are important for protein folding at normal temperatures, it is assumed that one role of heat shock proteins in the development of thermotolerance is to refold proteins whose structure is disturbed by the heat shock (Linsquist and Craig, 1988; Hartl, 1996). Our data show that CG32130, *starvin*, is expressed in a similar manner to heat shock genes. The developmental expression of *starvin* and the effects of loss of function, failure to eat and grow, have been studied in detail (Coulson *et al.,* 2005). The same authors have identified a BAG domain in *starvin* and compared it to mammalian BAG domain proteins. These proteins bind to Hsp70 and regulate ATP turnover (Hohfeld and Jentsch, 1997; Takayama *et al.*, 1997). CG32130 protein has also been shown to bind to Hsp70s and Hsc70 in *Drosophila* (Giot *et al.,* 2003), suggesting that it may have a similar function to mammalian BAG proteins.

Materials and Methods

Fly strains used were F36a and Oregon R wild type originally obtained from the CalTech collection.

Microarrays were done using the original set of *Drosophila* Genome chips from Affymetrix. Heterozygote (f^{36a}/+) female dorsal thoraces were dissected from 60 to 100 pupae at 34 hours of pupal development, the sensitive period for *forked* phenocopy induction. Pupae of this age were also heat shocked and either dissected immediately or allowed to recover for 10 hours before dissection. RNA preparation was done using the Tri-reagent and Rnease mini kit (Qiagen). cDNA synthesis, amplification, hybridization, and initial analysis for the microarrays were done according to the Affymetrix protocol at the Microarray Core Facility at the University of Colorado Health Science Center in Denver, CO. The cell file data from the microarrays was analyzed by N.P. using Bioconductor RMA (robust multi-array analysis) program from bioconductor.org.

For real time PCR, RNA was extracted as above from whole wild type larvae, pupae and adults that had been heat shocked under a variety of different conditions. The cDNA synthesis was done using TaqMan Reverse transcription reagents (Applied Biosystens). Real time PCR analysis was done at the Macromolecular Core Facility at the University of Wyoming using the SYBR green kit from Qiagen. PCR primers used were: Hsp 70, F1b- 5'-ttgggcaccttcgatctgt-3', R1- 5'-gcttcaccatgcgatcaatct-3'; CG32130, F1- 5'-gaatgtgaataggaggagctgg-3', R1- 5'-tgtggtgtgtgtgtgtgtgt-3'; DnaJ-1, F1- 5'-cgcattcgctgttcaaacg-3', R1- 5'-gttgtggtgggcttgatga-3'.

Results

Our microarray experiment was designed to look at gene expression in *Drosophila* pupae under conditions where a 40.5°C heat shock (HS) induces the *forked* phenocopy. The phenocopy can be prevented by a 30 minute 35°C thermotolerance-inducing heat shock immediately before the 40.5°C shock (TH, for thermotolerant) (Mitchell and Petersen, 1985; Petersen and Mitchell, 1988). We looked at gene expression immediately following each heat shock and 10 hours later as well as in control 34h pupae. The 10 hour recovery time was chosen based on our understanding of the recovery of protein synthesis in previous experiments (Petersen and Mitchell, 1981, 1982). In order to look at increases in gene expression in the thermotolerant state, we calculated the ratio of the gene expression value immediately after the thermotolerant heat shock (TH0) and gene expression value immediately following abrupt heat shock (HS0) for each gene. Table 1 is the result of sorting our data by the TH0 to HS0 ratio. This table shows the raw output of our RMA analysis of our *Drosophila* Affymetrix data for the first nine genes on the list. Because the thermotolerant pupae were heated at 35°C to induce thermotolerance and heat shock protein synthesis, we expected to see large increases in heat shock mRNAs, and this is the case. There are three genes that have not been previously identified as heat shock genes in this table. Of these, the most highly expressed after heat shock is CG32130, which we initially identified as a BAG gene using the affy target sequence to BLAST the non-redundant nucleotide database. Two other up regulated genes, which we have not followed up, are CG5290, which has prenyltransferase homology, and CG3428, which may have an F-box. While the amounts of RNA can be compared for the same gene under different conditions,

Table 1. This is the list of expression values in arbitrary units from the Affymetrix microarray experiment for the genes whose expression is most increased immediately following a 35-40.5°C heat shock as compared to a 40.5°C heat shock. 34h is RNA isolated from dorsal thoraces at 34 hours of pupal development with no heat shock. HS0 is RNA isolated from the same tissue following a 30 minute treatment at 40.5°C; HS10 is the same heat shock with a 10 hour recovery period at 25°C before dissection and RNA isolation. TH0 is RNA isolated from the same tissue following a double heat shock, 35°C for 30 minutes followed by 40.5°C for 30 minutes; TH10 is the same as TH0 except that the pupae were allowed to recover for 10 hours at 25 degrees before dissection. The ratio of THO to HS0 was calculated for each gene and the data was sorted by that ratio. Probe set ID is the Affymetrix number given to the set of oligonucleotides, usually 14-20, that are designed to hybridize with the target sequence for each gene.

Ratio TH0:HS0	Gene Symbol	34h	HS0	HS10	TH0	TH10	Probe Set ID
100.7705789	Hsp70Bc	647.0596	120.9986	5222.897	12193.1	2776.389	149782_at
69.4602381	Hsp68	184.5536	98.35646	5520.098	6831.863	2304.046	143197_at
13.73053265	DnaJ-1	646.9762	327.2157	3285.198	4492.846	1446.344	143835_at
10.95886298	CG32130	168.6312	111.9317	729.3557	1226.644	339.5332	151834_at
8.444642878	CG5290	29.91847	30.48848	91.11778	257.4643	39.53224	141652_at
6.463962704	Hsp70Bbb	94.88164	71.10796	688.7056	459.6392	301.4211	151036_f_at
4.382939012	CG3428	57.40421	43.63796	64.09633	191.2625	67.67389	154694_at
3.532894701	Hsp83	2370.656	2154.899	5062.813	7613.03	2852.281	143198_at

there is no precise way to compare RNA quantity between genes. However, it is obvious from the numbers in the table that Hsp70 is the most highly expressed heat shock gene and that CG32130 is expressed at a significantly lower level, which helps explain why it has not been previously identified as encoding a heat shock protein.

To confirm the data from the microarray analysis, we did real time PCR experiments on pupae of different ages as well as adults, larvae and cell lines under normal and heat shock conditions. In each case CG32130 expression increased significantly. The increase in expression of CG32130 following heat shock varies between three and ten fold which is similar to other heat shock proteins. The variability of the ratio is due at least in part to different levels of expression at 25°C at different times in development and in different tissues (Krebs and Feder, 1997). The maximum level of heat shock gene expression is also exquisitely sensitive to the time and temperature of lethal or phenocopy inducing heat shock, usually 40°C for 25-30 minutes for larvae and between 40 and 41°C for pupae depending on their precise age (Mitchell and Petersen, 1982). Figure 1 shows the results of three representative real-time PCR experiments done at different times, by different technicians, which confirm

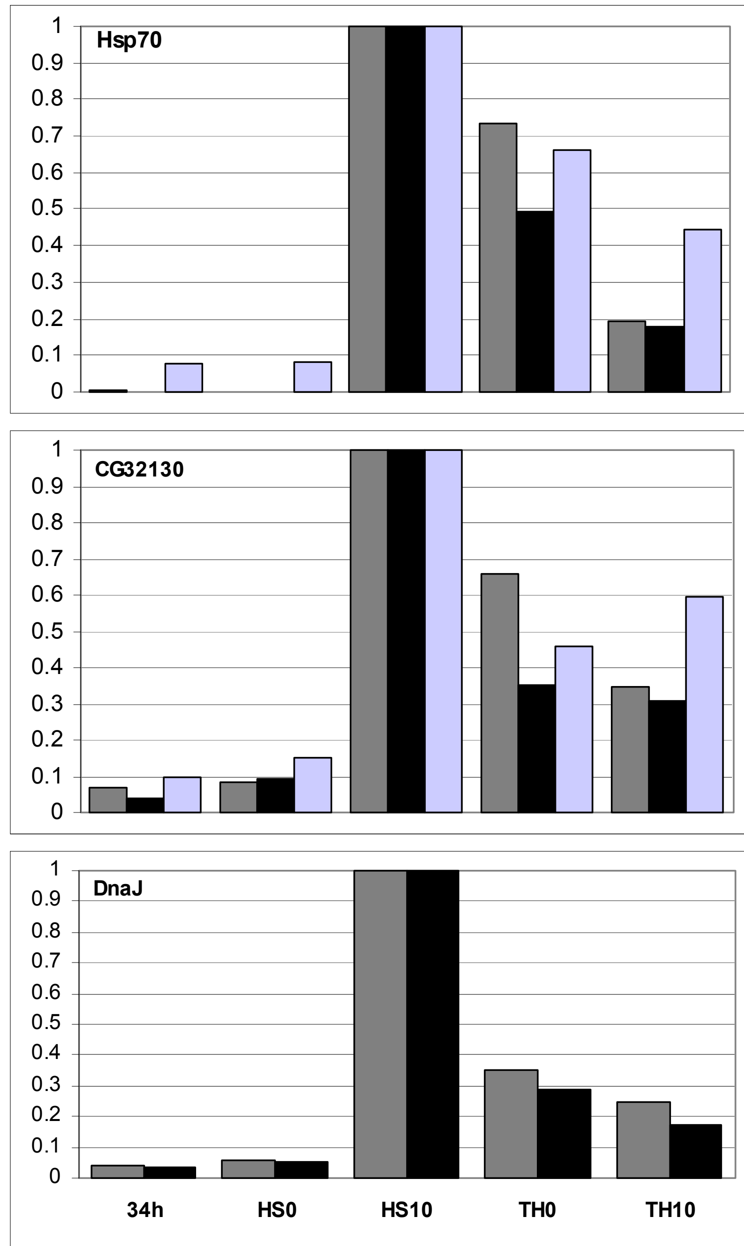

Figure 1. The gene expression profile for CG32130 is similar to that of Hsp70 and DnaJ. Real time PCR data showing levels of gene expression for three different experiments. Dark gray bars, 10-27-04; black bars, 11-1-04; light gray bars, 1-20-05. The expression values are normalized to the highest value. 34h indicates RNA isolated from whole pupae at 34 hours of pupal development with no heat shock. HS0 indicates RNA isolated from 34h following a 30 minute treatment at 40.4°C; HS10 is the same heat shock with a 10 hour recovery period at 25°C before dissection and RNA isolation. TH0 indicates RNA isolated from the same tissue following a double heat shock, 35°C for 35 minutes followed by 40.4°C for 30 minutes; TH10 is the same as TH0 except that the pupae were allowed to recover for 10 hours at 25°C before dissection.

that CG32130 is up regulated in 34 hour pupae in a pattern similar to both Hsp70 and Hsp67. The expression of each of these genes is low during normal development, increases following exposure to 35°C in the thermotolerant animals. The 40.5°C heat shock inhibits all mRNA and protein synthesis for a time and the delay in recovery results in heat shock gene expression being very high ten hours after a single heat shock (Petersen and Mitchell, 1982). As the recovery process proceeds in thermotolerant animals, the amount of heat shock mRNA and heat shock protein synthesis decreases. As in the microarray data, the amounts of PCR product for the same gene can be compared under different conditions, but comparing the amount of PCR product in different genes is not quantitative. In order to compare the patterns of gene expression we normalized the amount of gene expression to the largest value, HS10. By comparing these profiles, it can be seen that CG32130 behaves very much like Hsp70 and DnaJ, two classic heat shock genes. One difference may be in the thermotolerant recovery period, where CG32130 expression is more variable and may persist longer than that of Hsp70 and DnaJ.

Discussion

The expression of CG32130, *starvin*, under thermotolerance inducing conditions along with Hsp70 and DnaJ (which encodes another Hsp70 regulatory protein), suggests that it may function with Hsp70 in the development of thermotolerance. CG32130 contains a BAG domain and, since BAG domain proteins have been studied extensively in mammalian cell lines, it is useful to look at the mammalian literature for hints about how CG32130 might be involved in acquisition of thermotolerance. Human BAG-1 (Bcl-2-associated athanogene) was initially isolated as BCL2 associated protein in a human lymphoid cell line (Takayama *et al.,* 1995). However, it was soon found that BAG proteins bind to Hsp70 and function to increase the rate of ATP hydrolysis (Hohfeld and Jentsch, 1997; Takayama *et al.,* 1997). In mammals, heat shock causes apoptosis and BAG gene over expression can prevent apoptosis in mammalian cell lines (Takayama and Reed, 2001; Townsend *et al.,* 2004). Heat shock gene expression in mammalian cell lines can also prevent apoptosis, although the exact mechanisms involved in this protective effect are controversial (Jaattela *et al.*, 1997; Mosser *et al.,* 2000; Samali *et al.*, 2001; Steel *et al.*, 2004; Ueda *et al.*, 2004). The *Drosophila* BAG protein, CG32130, also binds to Hsp and Hsc proteins in *Drosophila* (Giot *et al.*, 2003.). This, along with the mammalian data, hints that in *Drosophila* Hsp70 in association with CG32130 protein could be involved in rearranging protein complexes in a way that prevents heat shock induced apoptosis.

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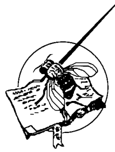

A significant increase in the rate of new deleterious mutations following interspecies crowding of *Drosophila melanogaster* by *Drosophila simulans.*

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Introduction

The possibility that interspecies crowding may significantly increase mutation rates is of importance to the breeding of captive endangered species and in estimating the effects of the introduction of invasive species. An increase in mutation rate due to the stress of interspecies crowding could decrease a species' chances for survival due to the accumulation of deleterious alleles. Likewise, captive endangered animals are often kept in close quarters with other species and are subjected to inbreeding. Several studies have indicated that exposure to environmental stress may increase the effects of inbreeding depression, though the exact factors causing the increase are unclear (Gaggiotti and Hanski, 2004; Keller *et al*., 2002). An increase in the rate of new deleterious mutations could be a factor by reducing the fitness of captive bred animals beyond that expected from inbreeding depression alone (Frankham, 2000).

Interspecies competition for limited resources has been shown to reduce native populations and, in some cases, force the native species out entirely (Human and Gordon, 2004; Tsutsui *et al*., 2000; Wauters *et al*., 2005). Thus, the presence of an invasive species can be seen as a stressor to the native population. Many environmental stressors have been shown to increase the mutation rate in *Drosophila melanogaster* (Hoffman and Parsons, 1997; Hoffman and Parsons, 1992). These stressors include starvation, heat, cold and larval crowding (Imasheva and Bubliy, 2003; Joshi, 1998; Lingren, 1972). The reported rate of increase is, however, variable and not always significant (Charlesworth *et al*., 2004; Chavarrias *et al*., 2001; Drake *et al*., 1998; Fry, 2001; Fry *et al*., 1999; Houle *et al*., 1992). The effect of interspecies crowding on mutation rate has not specifically been considered and may be especially relevant to current conservation efforts due to habitat compression, captive breeding and invasive species.

One important challenge is to determine if there are significant changes in mutation rates in stressed and unstressed populations. One possible way to meet this challenge is to employ mutation accumulation experiments. Mutation accumulation experiments have long been used to calculate deleterious mutation rates, but one problem that persists in their use is that of establishing suitable controls (Drake *et al*., 1998). The Binscy assay described here eliminates this problem through the use of concomitant sibling controls (Gong *et al*., 2005). To measure the influence of interspecies crowding on the mutation rate, we tested for the accumulation of new deleterious and lethal

mutations on the X chromosome of *D. melanogaster*, in the presence of *D. simulans*, by the use of the Binscy assay and compared these rates to those of *D. melanogaster* raised in the absence of *D. simulans.*

Materials and Methods

In the synthesis of the Binscy assay stocks we used a mating scheme modified from Muller and Oster (1963) (see Figure 1; Gong, *et al.*, 2005, for a discussion of this mating scheme). Binscy is a balancer X chromosome with the *B* (Bar eyes, dominant) and *y* (yellow body, recessive) mutations, plus multiple inversions that eliminate X-chromosome recombination in females. Gong *et al.* (2005) confirmed the balancing ability of the Binscy X chromosome by observing no recombination among 622 progeny from *w m f* / Binscy females as opposed to 206 recombinants recovered among 584 progeny from *w m f* / + + + females. C(1;Y^S)*oc ptg* is a combination of the X and the short arm of the Y chromosome, with the *oc* (ocelliless female and homozygous female sterile) and the *ptg* (pentagon, thoracic trident dark) recessive mutations. RY^L is the long arm of the Y chromosome in the shape of a ring. The Binscy/RY^L males are sterile because of the missing male fertility factors on the short arm of the Y chromosome, and C(1;Y^S)*oc ptg*/ C(1;Y^S)*oc ptg* females are sterile because of the homozygous *oc* mutation. For each generation, one Binscy/ C(1;Y^S)*oc ptg* female was mated with one C(1;Y^S)*oc ptg*/RY^L male (Figure 1). New deleterious mutations will accumulate over generations on the Binscy balancer X chromosome in the Binscy/ C(1;Y^S)*oc ptg* females. Since C(1;Y^S)*oc ptg* homozygous females and Binscy/RY^L males from this mating scheme are sterile, new deleterious mutations that occur on the X chromosome of the C(1;Y^S)*oc ptg*/RY^L males are in a hemizygous state and are eliminated by selection. To the extent that deleterious mutations might temporarily be present on the C(1;Y^S) *oc ptg*, the calculation of U_d (diploid mutation rate) will be decreased. However, deleterious mutations that occur on the Binscy X chromosome in Binscy/ C(1;Y^S)*oc ptg* females are buffered from selection, because mutations are maintained as heterozygotes against wild-type alleles on the C(1;Y^S)*oc ptg* chromosome. Consequently, over time,

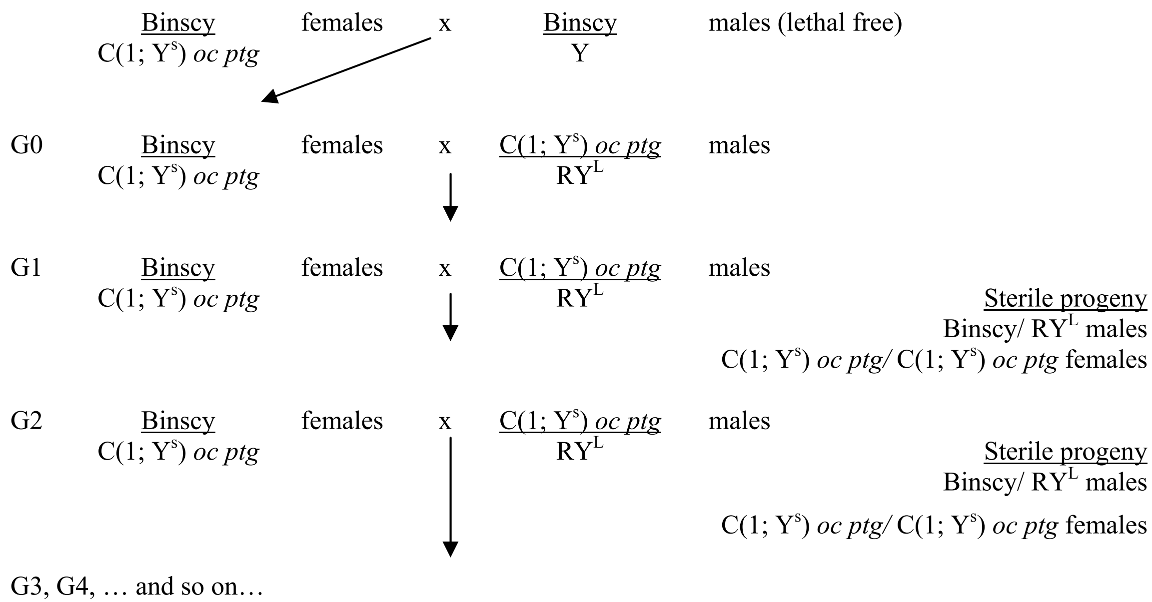

Figure 1. The mating scheme for the accumulation of deleterious mutations in the Binscy X chromosome of *Drosophila melanogaster*. In every generation (G0, G1, G2… etc.) one female and one male are randomly selected to mate.

deleterious mutations will accumulate on the Binscy chromosome but are not expected to accumulate on the $C(1;Y^S)$*oc ptg* chromosome. By comparing the viability of Binscy/RY^L males (where mutations are accumulating) to the viability of $C(1;Y^S)$*oc ptg*/RY^L males (where mutations are not accumulating), the rate of new mutations on the X chromosome can be determined using the method of Bateman (1959) and Mukai (1964).

The deleterious genomic mutation rate was calculated using the method of Gong *et al.* (2005) that followed the Bateman-Mukai technique (Bateman, 1959; Drake *et al.,* 1998; Lynch and Walsh, 1998; Mukai, 1964). The accumulation of deleterious mutations over time will lead to a predicted, steady reduction in mean viability and an increase in variance between lines. The rate of decline in mean variability (ΔM) and the rate of increase in variance of viability among lines (ΔV) can be estimated by regression analysis. If one assumes that spontaneous mutations are distributed on the X chromosome according to a Poisson distribution, ΔM and ΔV can be expressed as:

$$\Delta M = M_s U_x$$

$$\Delta V = (M_s^2 + V_s)U_x$$

Rearranging these two equations gives

$$U_x = \Delta M^2 / \Delta V,$$

where M_s and V_s are the mean and variance of s (the effect of a mutation on viability) and U_x is the mean number of deleterious mutations on the X chromosome in one generation. Since the Binscy assay estimates the haploid mutation rate using the X chromosome and the X chromosome contains about 15.97% of the genes in the haploid genome in *D. melanogaster,* the estimated diploid mutation rate (U_d) is two times U_x, divided by 15.97% (Gong *et al.,* 2005).

In addition to deleterious mutations, a new lethal mutation on the Binscy X chromosome was counted when a line had no Binscy/RY^L males for three generations or more. The summation of the total number of lines screened in each generation is the total number of Binscy X chromosomes assayed. Division of the number of lines showing no Binscy/RY^L males by the total number of lines assayed gives the new lethal mutation rate.

One hundred initial lines were created, each from a single $C(1;Y^S)$*oc ptg*/ RY^L male and a single Binscy/ $C(1;Y^S)$*oc ptg* female. The initial pairs were placed in vials along with three male/female pairs of w^{pch} *D. simulans* of the same age. The w^{pch} flies exhibit a peach colored eye making it easy to distinguish between *D. simulans* and *D. melanogaster* when scoring. For each generation, vials were established as above with one pair of *D. melanogaster* and three pairs of *D. simulans* selected at random from the preceding generation. Progeny were then scored for deleterious and lethal mutations over the course of 17 generations. Only those lines viable at the end of the experiment were used in calculating the final result for deleterious mutations. One hundred control vials, each containing one $C(1;Y^S)$*oc ptg*/ RY^L male and one Binscy/ $C(1;Y^S)$*oc ptg* female, were also established from the same stocks but without the addition of *D. simulans.*

Results and Discussion

The rate of new lethal mutations did not differ significantly between the crowded and the uncrowded populations over the course of the experiment (Table 1). The rate of new deleterious mutations did, however, increase significantly in the crowded populations (Table 2), giving a mutation rate higher than most in the literature (Garcia-Dorado *et al*., 1999). Regression analysis of the estimated diploid genomic mutation rate showed a significant increase in the rate of deleterious mutations in the crowded lines ($P = 0.024$, Figure 2). These latter results support the hypothesis that the presence of even a modest number of interspecies individuals might result in a significant amount of stress that may increase the mutation rate.

Table 1. Lethal mutation rate for interspecies crowded vs. uncrowded flies over 17 generations.

	Lethal Chromosomes	Total Chromosomes Scored	%[a]
Crowded Flies	8	924	0.0087
Control	2	903	0.0027

[a]P = 0.1

Table 2. Mutation rates of interspecies crowded flies v. non-crowded flies after 17 generations. ΔM = change in mean, ΔV = change in variance, U_x = estimated mutation rate for the X chromosome, U_d = estimated diploid genomic mutation rate.

	ΔM	ΔV	U_x	U_d[a]
Crowded Flies	0.0142	0.002344	0.085961	1.075858
Control	0.01065	0.002558	0.044335	0.55488

[a]P = 0.024

Charlesworth and Charlesworth (1999) have stated that a major cause of inbreeding depression may be from the accumulation of new, recessive deleterious mutations. If this is the case, then any increase in the mutation rate will result in a possible decrease in fitness greater than that predicted by inbreeding. This decrease in fitness could have a significant impact on the captive breeding of endangered species, their possible reintroduction to the wild and the effect of invasive species on native populations.

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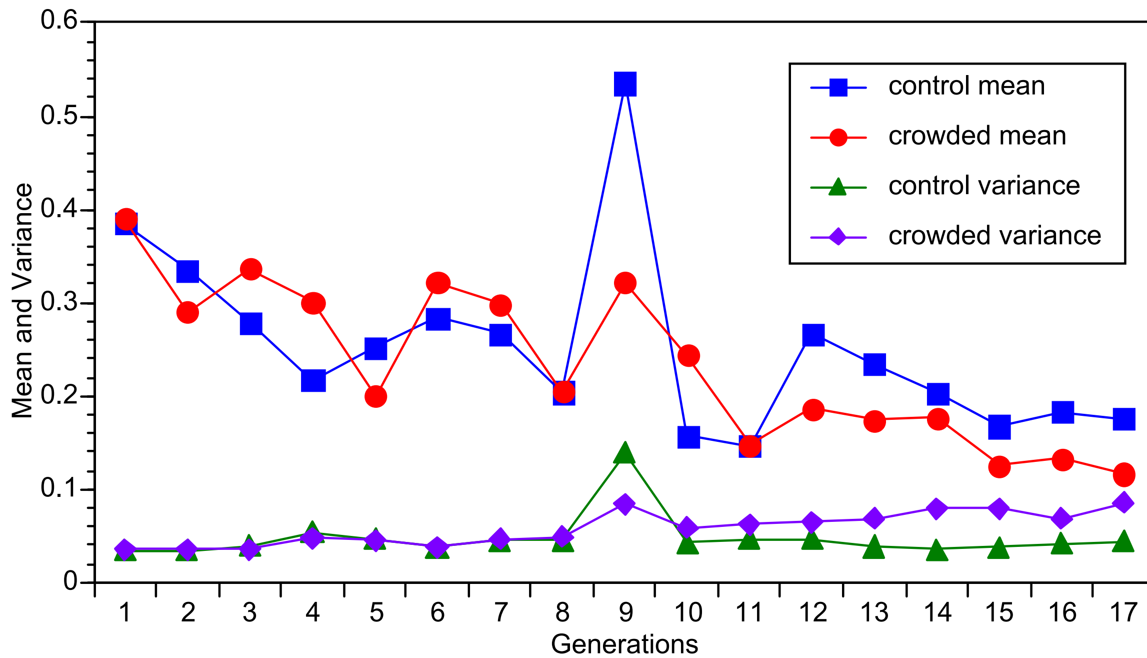

Figure 2. Comparison of mean and variance for interspecies crowded vs. uncrowded flies over 17 generations.

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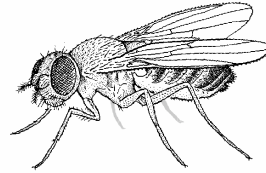

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