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Efeito de substâncias químicas de uso laboratorial sobre *Drosophila melanogaster* (Diptera Drosophilidae) do ponto de vista bioquímico e morfológico

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EFEITO DE SUBSTÂNCIAS QUÍMICAS DE USO LABORATORIAL SOBRE *Drosophila melanogaster* (Diptera – Drosophilidae) DO PONTO DE VISTA BIOQUÍMICO E FENOTÍPICO

Dissertação apresentada para a obtenção do Grau de Mestre em Biologia Animal no Curso de Pós-Graduação do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista – UNESP

Orientador: Prof. Dr. Gustavo Orlando Bonilla Rodriguez

Co-orientador: Prof. Dr. Carlos Roberto Ceron

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DISSERTAÇÃO APRESENTADA PARA OBTENÇÃO DO GRAU DE MESTRE EM BIOLOGIA ANIMAL

COMISSÃO JULGADORA

Presidente e Orientador:.....

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Dedico este trabalho:

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"Educar é um trabalho que consiste em trazer à tona a natureza oculta no ser humano. Consiste em reconhecer o ser humano como filho de Deus e acreditar firmemente na sua capacidade latente. A educação deve ter como base a fé no ser humano, a postura mental de contemplar algo sublime, invisível aos olhos físicos" do livro Shinjitsu o Motomete.

Obrigada pela constante dedicação e incentivo aos meus estudos

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"Muitos são os professores, poucos são os mestres. Os professores ensinam por palavras em templos, com os mestres aprendemos por ação e exemplos." Helena Pinheiro

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Aprendi...

" Aprendi que eu não posso exigir o amor de ninguém. Posso apenas dar boas razões para que gostem de mim e Ter paciência, para que a vida faça o resto.

Aprendi que não importa o quanto certas coisas sejam importantes para mim, tem gente que não dá a mínima e eu jamais conseguirei convencê-las.

Aprendi que posso passar anos construindo uma verdade e destruí-la em apenas alguns segundos.

Que posso usar o meu charme por apenas 15 minutos, depois disso, preciso saber do que estou falando.

Eu aprendi...Que posso fazer algo em um minuto e ter que responder por isso o resto da vida.

Que por mais que se corte uma pão em fatias, esse pão continua tendo duas faces, e o mesmo vale para tudo o que cortamos em nosso caminho.

Aprendi... Que vai demorar muito para me transformar na pessoa que quero ser, e devo ter paciência.

Mas, aprendi também que posso ir além dos limites que eu próprio coloquei.

Aprendi que preciso escolher entre controlar meus pensamentos ou ser controlado por eles.

Que os heróis são pessoas que fazem o que acham que devem fazer naquele momento, independentemente do medo que sente.

Aprendi que perdoar exige muita prática.

Que há muita gente que gosta de mim, mas não consegue expressar isso.

Aprendi... Que nos momentos mais difíceis, a ajuda veio justamente daquela pessoa que eu achava que iria tentar piorar as coisas.

Aprendi que posso ficar furioso, tenho o direito de me irritar, mas não tenho o direito de ser cruel.

Que jamais posso dizer a uma criança que seus sonhos são impossíveis, pois seria uma tragédia para o mundo se eu conseguisse convencê-la disso.

Eu aprendi que meu melhor amigo vai me machucar de vez em quando, e que eu tenho que me acostumar com isso.

Que não é o bastante ser perdoado pelos outros, eu preciso me perdoar primeiro.

Aprendi que, não importa o quanto meu coração esteja sofrendo, o mundo não vai parar por causa disso.

Eu aprendi... Que as circunstâncias de minha infância são responsáveis pelo que eu sou, mas não pelas escolhas que eu faço quando adulto;

Aprendi que numa briga preciso escolher de que lado eu estou, mesmo quando não quero me envolver.

Que, quando duas pessoas discutem, não significa que elas se odeiem; e quando duas pessoas não discutem não significa que elas se amem.

Aprendi que por mais que eu queira proteger os meus filhos, eles vão se machucar e eu também. Isso faz parte da vida.

Aprendi que a minha existência pode mudar para sempre, em poucas horas, por causa de gente que eu nunca vi antes.

Aprendi também que diplomas na parede não me fazem mais respeitável ou mais sábio.

Aprendi que as palavras de amor perdem o sentido, quando usadas sem critério.

E que amigos não são apenas para guardar no fundo do peito, mas para mostrar que são amigos.

Aprendi que certas pessoas vão embora da nossa vida de qualquer maneira, mesmo que desejemos retê-las para sempre.

Aprendi, afinal, que é difícil traçar uma linha entre ser gentil, não ferir as pessoas, e saber lutar pelas coisas em que acredito."

William Shakespeare

SUMÁRIO

Resumo

A presença de resíduos químicos no ambiente afeta todo o ecossistema. Uma das técnicas empregadas para o estudo dos efeitos tóxicos dos resíduos químicos é o biomonitoramento, que utiliza como modelos organismos vivos, entre eles os insetos. Um dos gêneros utilizados é a *Drosophila*, em particular a espécie *D. melanogaster* de distribuição cosmopolita. É um organismo geneticamente bem explorado, de fácil manutenção e rápido ciclo biológico, que após o seqüenciamento completo de seu genoma revelou homologia de 60% de seus genes com os genes de doenças humanas. Entre as substâncias químicas que foram investigadas neste trabalho, incluem-se três metais tóxicos (alumínio, cromo e chumbo), um corante (azul Coomassie brilliant G250) e o produto sintético acrilamida. A análise foi realizada em concentrações baixas (50μM) com ênfase na característica acumulativa de cada produto químico durante dez gerações de exposição. As análises, fundamentadas na biologia do inseto, avaliaram sua produtividade, alterações morfológicas, viabilidade das diversas fases do desenvolvimento, o peso dos insetos, o comportamento sexual e a atividade enzimática da carboxilesterase. Foi realizado também um experimento de produtividade comparativo com duas linhagens, uma massal e outra isofêmea. A exposição à acrilamida, aumentou a produtividade na primeira exposição, e também reduziu a viabilidade de ovos para adultos nas gerações F_3 e F_{10}. Por sua vez, entre os metais, o chumbo, afetou a viabilidade de ovos em pupas na décima geração e apresentou alterações no tempo de pré-cópula e de cópula. A exposição ao cloreto de alumínio, revelou na primeira e na quinta geração aumento do tempo de pré-cópula, e na décima geração aumento do tempo de cópula. O dicromato de potássio apresentou aumento da freqüência das alterações morfológicas na terceira geração. Houve redução do número de ovos nas

gerações F_3 e F_{10}, aumento do tempo de pré-cópula nas gerações F_1 e F_5 e de cópula na geração F_{10}. O corante azul Coomassie foi o produto que menos afetou a biologia da *D. melanogaster*. Na sétima geração houve aumento significante da emergência de adultos. Nos testes de comportamento foi observado aumento do tempo de pré-cópula com o passar das gerações, no entanto, houve aumento da viabilidade dos ovos quando a fêmea advinha do grupo tratado com Coomassie. Na comparação com as duas linhagens, cada uma apresentou suas peculiaridades, de acordo com o composto químico testado, a alteração no número médio de emergentes foi freqüente, mas não manteve um padrão ou tendência. O trabalho fez surgirem várias questões quanto à toxicidade e mecanismos de defesa que deverão ser analisados em maior profundidade.

INTRODUÇÃO, JUSTIFICATIVA E OBJETIVOS

Capítulo Um

I. INTRODUÇÃO

Os produtos químicos estão presentes em todas as formas de atividade humana, seja direta ou indiretamente. No entanto, dependendo das condições em que se encontram ou são utilizados, podem gerar riscos à saúde e ao meio ambiente. Uma das formas mais evidentes da exposição humana aos produtos químicos ocorre pela geração de resíduos decorrentes das atividades industriais (CALAFAT et al., 2006), sendo que estas muitas vezes são realizadas em locais próximos a residências, aumentando a probabilidade de contaminação da população pelos resíduos sólidos, líquidos e gasosos (JORDAENS et al., 2006).

Outra forma de exposição é a profissional, seja ela em indústrias de manufatura ou em laboratórios de pesquisa. Muitas vezes os processos desenvolvidos requerem contato direto com produtos tóxicos, carcinogênicos ou genotóxicos. Atualmente, para uma parte desses produtos, não há muita informação sobre os efeitos tóxicos à saúde, (CALAFAT et al., 2006), principalmente quando se refere à exposição contínua por um tempo maior.

Como a presença dos resíduos químicos no ambiente afeta todo o ecossistema, em termos de funcionamento e estrutura (FENT, 2003), durante os últimos 30 anos começaram a ser empregadas técnicas baseadas em biomarcação para a análise quanto à exposição aos tóxicos. O biomonitoramento como ferramenta de estudo de toxicidade apresenta diversas vantagens como baixo custo, possibilidade de registrar a poluição e seus efeitos mais longos em muitos locais simultaneamente (WANNAZ et al., 2005). Como essa técnica baseia-se na utilização de organismos vivos, recentemente, iniciou-se um processo de redução do uso de vertebrados nos testes e pesquisas toxicológicos (SIDDIQUE et al., 2005). Devido à importância ecológica dos invertebrados, seu uso

como sistema modelo para vertebrados (HIRSCH et al., 2003) tem contribuído para estudos de toxicologia.

Um invertebrado de grande utilização em estudos de toxicologia é a *Drosophila melanogaster,* de distribuição cosmopolita. O gênero *Drosophila* é recomendado pelo Centro Europeu de Validação de Métodos Alternativos (ECVAM), como inseto modelo apropriado para pesquisa e testes nessa área (BENFORD et al., 2000). Durante a última década, este gênero emergiu como um dos melhores organismos para estudos de doenças humanas e pesquisas toxicológicas (SIDDIQUE et al., 2005). Dessa forma, tem sido utilizado não só porque as populações naturais são resistentes às toxinas lançadas pelos humanos no ambiente, mas também devido às vantagens advindas de seu ciclo biológico, como o rápido desenvolvimento e a fácil manipulação.

O ciclo biológico de *Drosophila melanogaster* consiste de três estágios larvais, diferenciados em 1º, 2º e 3º instars, seguidos de um estágio que constitui a forma pupal, a qual após cinco dias, emerge na forma de adulto voador. Dessa forma, uma geração completa-se dentro de duas semanas. Outras vantagens do uso de *D. melanogaster* que merecem atenção especial são entre outras, a facilidade de cruzamento, pequeno número de cromossomos, a possibilidade de trabalhar com grande número de indivíduos, além dos benefícios advindos da simplicidade no processo de transformação genética e a disponibilidade de sua seqüência genômica completa (WILSON, 2001), a qual revelou que cerca de 60% dos genes humanos possuem homologia com os de *Drosophila* (SCHNEIDER, 2000). Por volta da segunda década do século passado Lutz e Sturtevant concluíram que a *Drosophila* pode servir não só para estudos genéticos em comparações com animais superiores, como também para investigações de comportamento e seleção sexual, uma vez que nessas espécies o comportamento de cópula é espécie-específico (ASHBURNER et al.,1983). Somado a todas essas

vantagens, a *Drosophila* é um sistema conveniente para responder questões de como o organismo se defende contra o excesso de um determinado poluente, uma vez que muitos aspectos da homeostase, principalmente dos metais, são conservados entre as moscas e os humanos (YEPISKOPOSYAN et al., 2006).

Alguns resíduos tóxicos, principalmente das indústrias, são potencialmente perigosos porque contêm metais pesados e corantes (SECO et al., 2003). Os corantes têm origem orgânica e são de difícil degradação e fácil bioacumulação nas cadeias alimentares (TIEDGE et al., 1986). Nas águas residuárias ou mesmo nos vapores industriais ainda podem ser encontradas substâncias de origem sintética, cuja toxicidade pode ser decorrente da inalação ou mesmo do contato com a pele (CAMARGO et al., 2002).

Acredita-se que os metais sejam os agentes tóxicos mais conhecidos pelo homem, diferindo dos demais porque não são sintetizados nem destruídos pelo homem. A atividade industrial diminui significativamente a permanência desses metais nos minérios, produzindo novos compostos, e alterando a distribuição desses elementos no planeta.

Muitos íons são vitais para os processos biológicos como a transcrição, respiração e crescimento. Entretanto, o acúmulo de metais essenciais como cobre e zinco ou não essenciais como cádmio e mercúrio é tóxico ao organismo (YEPISKOPOSYAN et al., 2006). Em termos de terminologia química, esses compostos são chamados também de “metais tóxicos”, são elementos estáveis, não podem ser metabolizados pelo organismo, não possuem função fisiológica e são bioacumulativos na cadeia alimentar. Nesta lista inclui-se: mercúrio, níquel, chumbo, arsênio, cádmio, alumínio, platina e cobre. Chegam ao organismo via inalação, ingestão e absorção pela pele, e se acumulam nos tecidos em ritmo mais rápido do que os

processos de detoxificação e eliminação do corpo. A exposição dos humanos a essas substâncias tem crescido dramaticamente nos últimos 50 anos como resultado de um aumento exponencial no seu uso em processos industriais e seus derivados (MERCÚRIO – UM METAL TÓXICO..., 2007).

Entre os danos produzidos pelos metais tóxicos, estão aqueles derivados da proliferação dos radicais livres oxidativos que eles mesmos causam. Segundo estudos, duas das maiores enzimas antioxidativas inibidas por alguns dos metais pesados são a superóxido dismutase e a catalase. (THE BODY HAS NEED FOR…, 2006). Outra análise da toxicidade do oxigênio em sistemas vivos envolve diretamente a oxidação de grupos tiólicos das enzimas, e a produção de intermediários tóxicos como o peróxido de hidrogênio e radical hidroxila. O tripeptídeo glutationa evita a oxidação das enzimas, enquanto que a peroxidase e catalase são enzimas efetivas em manter os baixos níveis de peróxido de hidrogênio. A superóxido dismutase converte o ânion superóxido em uma reação desproporcional de peróxido de hidrogênio e oxigênio e pode indiretamente prevenir a formação de íon ferroso ou do oxi-radical orgânico (NICKLA et al., 1983).

Os metais ainda provocam danos neuronais irreversíveis no desenvolvimento dos mamíferos através de mecanismos de ação desconhecidos (AKINS et al., 1992). Podem também de forma direta (através de mutação na célula germinal) ou indireta (via mutação somática, ou efeitos fisiológicos e ecológicos) mudar a arquitetura genética da população (GILLESPIE e GUTTMAN, 1999; BICKHAM et al.,2000; DE WOLF, BLUST e BACKLJAU, 2004). Entre as respostas bioquímicas da contaminação por metais estudadas nos vertebrados foram focalizados os estudos com metalotioneínas, proteínas pequenas, ricas em cisteínas, encontradas em vários tecidos, responsáveis pela ligação dos íons metálicos a fim de facilitar o processo de detoxificação (WILSON, 2001).

Outras enzimas extensivamente estudadas em *Drosophila* compõem o grupo das esterases. Estas estão entre as enzimas geneticamente variáveis encontradas tanto em plantas como em animais. Um dos grupos das esterases é constituído pelas carboxilesterases (E.C. 3.1.1.1), as quais são altamente polimórficas em *Drosophila*. Esse grupo de enzimas hidrolisa ésteres de cadeia curta de ácidos graxos, possuindo diversas funções que incluem a neurotransmissão em animais, degradação de feromônios, de xenobióticos no fígado de mamíferos, de organofosforados e de outros xenobióticos e especificamente, resistência à inseticida em insetos (BONACCI et al., 2004).

Com relação aos estudos de toxicidade, em termos de biomonitoramento e ecotoxicologia, é importante lembrar que existem várias formas de resistência à exposição de toxinas. O organismo pode evitar as toxinas por vias comportamentais, envolvendo, neste caso, mudanças genéticas, entretanto, esse mecanismo não é muito relevante. As toxinas podem, também, penetrar no inseto através da cutícula, pelo sistema respiratório ou ainda pelo sistema digestório. Essas mudanças nas rotas de entrada podem resultar no desenvolvimento de mecanismos de resistência; isto devido à complexa estrutura de entrada de compostos no organismo. No entanto, uma mudança em só um componente bioquímico pode não ser suficiente para impedir a penetração da toxina (WILSON, 2001).

O mecanismo de maior interesse e eficácia, em termos de resistência dos animais, talvez seja o relacionado às alterações metabólicas causadas pelas toxinas. Usando uma variedade de reações enzimáticas, os organismos possuem a capacidade de metabolizar uma grande diversidade de moléculas orgânicas, normalmente tornando-as mais hidrofílicas (e geralmente menos tóxicas) e assim, mais facilmente excretáveis pelo corpo. Esta habilidade evoluiu para desintoxicar fitotoxinas da comida, e os

insetos, assim como os demais animais, usam o mesmo conjunto de enzimas para metabolizar os xenobióticos encontrados no ambiente. Outro tipo de resistência a toxinas é caracterizada pela interação com moléculas alvo. Este mecanismo constitui-se o segundo maior encontrado nos insetos. Uma vez no interior do inseto, as toxinas interagem com algumas moléculas ditas alvo, tipicamente proteínas, e alteram sua função, o que resulta em diversas patologias (WILSON, 2001).

Assim, os efeitos inter-individuais das drogas e xenobióticos estão baseados tanto nos fatores fisiopatológicos e interações ambientais, como nas características genéticas. Em muitos casos a toxicidade depende da concentração no sítio de ação e também das reações advindas do produto tóxico (CASCORBI, 2006).

II. JUSTIFICATIVA

A exposição aos insumos químicos é cada vez mais proeminente, estando estes presentes no ar, no solo e ainda nos alimentos e na água. A análise desta exposição associada aos conhecimentos relativos à saúde, permite estabelecer os riscos químicos aos quais está exposta a população, e possibilita uma intervenção efetiva a fim de se compreender os efeitos reais dos poluentes, além de prevenir uma provável contaminação. Torna-se, assim, essencial o estudo dos efeitos tóxicos decorrentes da exposição crônica a poluentes, bem como dos mecanismos de defesa disponíveis aos seres vivos.

III. OBJETIVOS

O presente trabalho teve por objetivo determinar o efeito potencialmente tóxico de algumas substâncias químicas encontradas em especial nos laboratórios de pesquisa, utilizando-se a *Drosophila melanogaster*, como bioindicador. A determinação da toxicidade foi realizada para os seguintes metais e produtos químicos: alumínio (na forma de cloreto de alumínio), cromo (dicromato de potássio), chumbo (nitrato de chumbo), acrilamida e azul de coomassie brilliant blue G-250.

A análise, fundamentada na biologia da mosca, teve por objetivo estudar, por 10 gerações e em duas linhagens, (uma isofêmea e outra massal) os aspectos abaixo relacionados, sendo os produtos químicos ou metais utilizados na concentração de 50 μM. :

- a produtividade diária ao longo de 15 dias,
- o comportamento em termos dos tempos de pré-cópula e cópula,
- alterações nos padrões morfológicos (no padrão de tergitos e coloração, de posicionamento de estruturas corpóreas, peso, entre outros).

- A nível bioquímico, com as medidas de atividade enzimática da carboxilesterase.
- Foi feita, ainda a análise do efeito de diferentes concentrações das substâncias químicas testadas.

Effects of the exposure of *Drosophila melanogaster* (Diptera – Drosophilidae) to Coomassie Blue

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Capítulo Dois

Effects of the Exposure of *Drosophila melanogaster* (Diptera - Drosophilidae) to Coomassie Blue.

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INTRODUCTION

The substantial increase in the generation of industrial solid wastes, is of great concern for scientists all over the world and may affect not only particular species of flora and fauna, but also the structure and function of entire ecosystems (Fent, 2003). Information on risks to human health due to exposure to environmental chemicals is a partially unexplored area, and biomonitoring programs are useful methods for investigating in this area (Calafat et al., 2006).

In the recent years, there has been an effort to reduce the use of higher animals in toxicological research and testing (Siddique et al., 2005). Emphasis has focused in holometabolous insects, which have a complex life cycle with different stages. Larvae and adults have different shape, and may have a different alimentary regime. Similar environmental stresses can affect each life stage in a different manner, and the adaptive strategies may depend on a particular life stage (Loeshche et al., 1996).

Drosophila melanogaster is a well-established insect model, recommended by the European Center for the validation of Alternative Methods (ECVAM). This species has been used extensively for studies in genetics and developmental biology (Siddique et al., 2005), and over the last decade, *Drosophila* has emerged as one of the most powerful models for human diseases (Auluck et al., 2002; Kaazantsev et al., 2002) and for toxicological research (Siddique et al., 2005). This fruit fly has several advantages, such as a short life cycle (one generation is complete within two weeks) and easiness to perform genetic crossing studies (Wilson, 2001).

Solid wastes generated by some industries are hazardous because they may contain heavy metals and pigments. The dyes are aromatic compounds, constituted by lignocellulosic material, that can be degraded by microorganisms (Balan and Monteiro, 2001). However, the inconvenient is the fact that organic and inorganic substances are difficult to degrade (Anliker et al., 1981). The literature reveals that the dyes have high potential to accumulate in the alimentary chain (Tonogai et al., 1980, Tiedge et al., 1986).

Chemical industries and laboratories dispose great volumes of those compounds in the sewage system. Among pigments, Coomassie blue is one of the most used dyes in

biological laboratories, both for protein measurement and to stain electrophoresis gels. The literature revels that in humans the ingestion of low doses of coomassie blue do not cause harmful effects; in higher doses can produce hyperesthesia of skin and muscle, nausea, vomiting and diarrhea (Hoffman, et al., 1961).

Although not considered as possessing moderate risks to health, this work intended to analyze the effect of Coomassie blue in *D. melanogaster.* We understand that it is important to determine the chemical concentrations that can produce damage in this organism.

MATERIALS AND METHODS

Specimens of *Drosophila melanogaster* were collected in São José do Rio Preto, State of São Paulo, Brazil, in May 2005. The individuals were maintained in vials with banana-agar medium at constant temperature at 25ºC. The females were used to set up a stock of an isofemale line homozygous.

To the culture medium used for feeding the insects we added Coomassie Brilliant blue G-250 at a final concentration 50 μM and the mixture was completely mixed. A control group was fed with uncontaminated culture medium.

A bioaccumulation study was performed for ten generations. Twelve virgin couples of females and males emerged from the stock were used to generate the first generation; for the other generations, 12 new couples were picked up among the animals emerged on the fifth day after the first adults emerged. The females were allowed to lay eggs for ten days. The emerged adults were counted every day for 9 days consecutively, and then new countings were done for the 11^{th} , 13^{th} and 15^{th} days. During the experiment, all the adults were also analyzed phenotypically (tergites color and pattern, wings shape and opening, and eyes' color).

In order to study the effects on the insects' weight, adults from F_{10} were separated by sex for six days consecutively, transferred to 1.5mL tubes, and were daily weighted using an analytical balance.

In the mating test, the adults from F_1 and F_{10} were separated for six days by sex in groups of five insects, and maintained at constant temperature (25º). After six days, the females and males were transferred, without anesthesia, to the same vial and observed for one hour to determine the time of pre-copulation and copulation.

For assays of enzymatic activities, we formed three samples, each one with five adults collected from F_5. The animals were homogenized in 100mM phosphate buffer pH6.2. Carboxylesterase activity was measured by the method from Ellman et al. (1961). Absorbance readings were performed in a Varian 100 spectrophotometer and all the assays were measured in triplicate. Protein concentration was determined according to Bradford (1976).

In the viability experiments a couple of adults from F_{10} was placed in a 250 mL bottle containing a plastic spoon filled with agar-sugar medium. The female was allowed to lay eggs for 24 hours, and then the couple was removed. The eggs were counted and the spoon was placed within a bottle containing medium culture. For couples picked up from a control group, the spoon was placed in a control bottle, doing a similar procedure for those exposed, transferring to a bottle containing Coomassie blue. Subsequently eggs, pupae and adults were counted.

The analyses of different concentrations were done for couples from F_8. One female and one male were allowed to couple for 24 hours in a vial with culture medium. The male was removed and the female was allowed to lay eggs for three days, when she was transferred to a new identical vial. The procedure was done for seven consecutive times, and as offspring was emerging, it was scored. Three replicates were done for each concentration and nine for the control group.

For the statistical analysis for bioaccumulative experiments the 2-proportions equality test (Z-normal approximation) (Moore, 2005) was carried out. The graphs were represented as the proportion of emerged flies emerged in three replicates in each day per flies emerged in that generation. In the other experiments, we performed the Student's *t* test (Zar, 1999). Differences were considered significant when $p<0.05$. The program BioEstat 4.0 (Ayres et al., 2005) was utilized.

RESULTS AND DISCUSSION

The variance among the groups was unequally distributed. For the isofemale strain six from the analyzed 10 generations (F_1, F_2, F_3, F_5, F_7 and F_9) showed a higher proportion of emergence for the exposed groups compared to the control. However, only for F_7 the differences were significant (Figure 1). For the exposed groups there was verified a tendency for a decreasing productivity starting at F_7, while for the control F_9 and F_{10} showed the same trend.

In a comparison between the massal (ML) and isofemale (IL) lines, in the massal strain only in the first generation the mean emergence for the treated group was higher than in the control, but in the others, the control group emergence was higher than for the exposed group. It could be a response for the product's presence, to increase the emergence in order to produce an adaptative offspring (Hirsch et al, 2003). For the isofemale strain in ten analyzed generations the emergence in treated group was always lesser than the control (Figure 2).

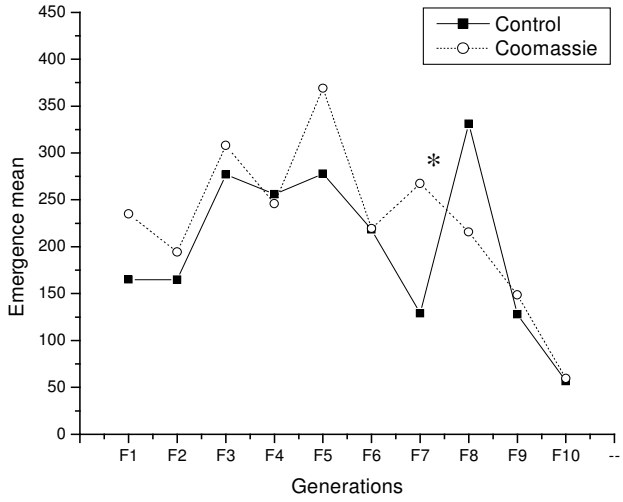
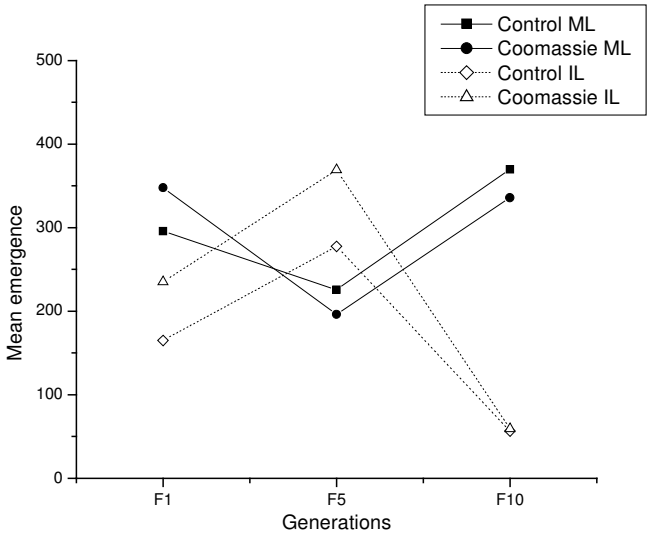


Figure 1: Graph of emergence proportion for each generation. * - indicates $p<0.05$.



for three generations, F_1, F_5 and F_{10}.

Pre-copulation and copulation times weres evaluated for F_1, F_5 and F_{10} generations (Figure 3). For pre-copulation time it was observed that Coomassie increased the period (the difference was not significant), whereas for copulation there was no difference between treated group and control, in the same analyzed generations. There is no evidence that coomassie can cause behavioral alterations. The literature reveals only physical indisposition in humans when the dye was ingested (Hoffman et al.,1961).

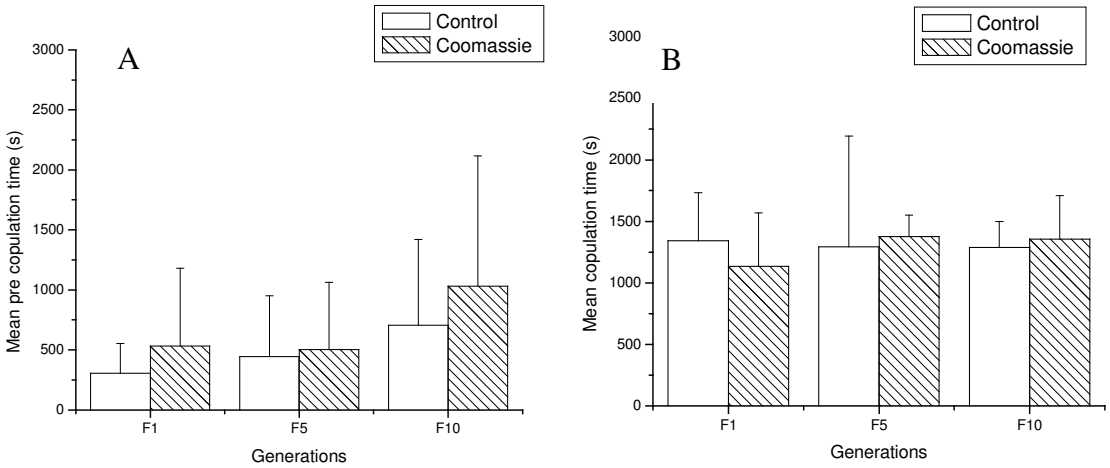


Figure 3: Behavioral tests for *D. melanogaster* exposed or not to Coomasie Blue. A: mean pre copulation time and B: mean copulation time

The influence of Coomassie on the flies weight, can be observed in figure 4. The mean weight of adults in submitted to Coomassie treatment, is about 3 times lower than those from the control in F_{10}. The effect on the insect weight could be explained by the hypothesis that adults avoid the medium culture because of the presence of the

contaminant, as proposed by Trumble et al. (2004). Other possibility is due to metabolic alterations, observed in some situations as acrylamide for example (Yousef et al., 2006).

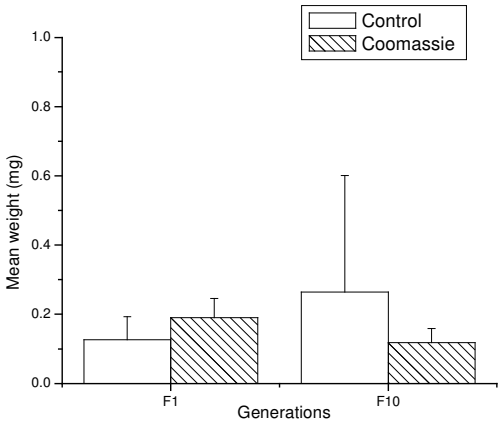


Figure 4: Graph for mean weight of *D. melanogaster* males and females in F_1 and F_{10}.

The egg viability experiments were conducted in F_3 and F_{10} generations (Table 1). Results show that in presence of coomassie there is increase in the egg and pupae viability in F_{10} generation as compared to control.

Table 1: Mean value and standard deviation in control and coomassie treated groups concerning eggs, pupae and adults scored in F3 and F10.

	Control				Coomassie			
	F3		F10		F3		F10	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Egg	47	12.5	21.5	2.5	53	13	8.5	0.5
Pupae	46	9.5	16.5	0.5	52.5	13.5	8	1
Adults	46	9	15.5	1.5	52.5	13.5	7.5	0.5
Viability Egg-adult (%)	97.9	5.6	72.3	1.4	98.8	1.3	88.2	0.7
Viability Egg-pupal (%)	100	30.3	77.3	6.7	98.8	1.3	93.8	6.3
Viability Pupal-adult (%)	100	94	93.8	6.3	100	0	94.4	5.6

Concerning enzymatic activity, carboxylesterase was analyzed in F5; but there were no differences between the control 72.5±23.2 U/mg) and the exposed group (70.6±24.6 U/mg). According to Bonnacci et al., (2004) this enzyme plays a prominent protection against neurotoxic compounds and in our experiments the coomassie exposition did not reveal an effect in behavioral tests.

For the concentration effect in the emergence of *D. melanogaster*, it was verified that in the lowest concentration the emergence was the highest one, and in this way there was not a dose-dependent response (Figure 5). Another characteristic of coomassie is its

rapid elimination in humans; after 8 hours it is completely excreted from the organism (Hoffman et al., 1961).

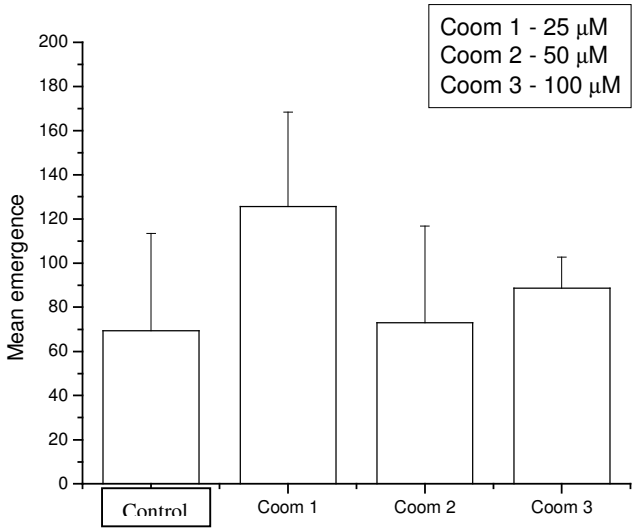


Figure 5: Mean emergence from F8 flies submitted to different acrylamide concentrations.

This work, although showing some alterations induced by the dye, corroborates the small toxicity when used in low concentrations. The solution used to stain electrophoretic gels is almost 50 fold more concentrated than that that we used, but our concern is, in the first place, with possible biological effects due to chronic exposure at low doses. In summary our results confirm previous observation about the low toxicity of Coomassie brilliant blue G-250.

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Effects of acrylamide on productivity, behavior and weight in two stocks of *Drosophila melanogaster*

Este artigo será submetido para Archives of Insect Biochemistry and Physiology

Capítulo Três

Effects of acrylamide on productivity, behavior and weight in two stocks of *Drosophila melanogaster*

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Abstract

Acrylamide is used mainly in chemical industries and laboratories. It is neurotoxic to experimental animals and humans, and also it has mutagenic and carcinogenic effects. The present work used *D. melanogaster* as a bioindicator for acrylamide toxicity, first following up ten generations exposed to a low acrylamide concentration (50μM) mixed to a banana-agar culture medium, and also to varying acrylamide concentrations. We analyzed the effects in fertility (scored as daily offspring), reproductive behavior (time of pre-copulation and copulation) and weight, using an isofemale and a massal strain, both collected in São José do Rio Preto, Brazil. In summary, acrylamide caused disturbances in *D. melanogaster,* and when the concentration increased, the effects tended to increase too. Alterations were observed in the flies' behavior (increase of the pre-copulation time), others were verified in the life cycle affecting in different manners the eggs, pupae and adults. The effects verified on each line were different, and the results indicated harmful effects even at low acrylamide concentrations.

Introduction

Human exposure to environmental synthetic chemicals has changed considerably in the past decades. This period has witnessed major changes in diets, lifestyle, and social practices, some of which may have profound effects on human health (Sharpe and Irvine, 2006). The consequences of chronic exposure to these compounds are frequently unknown. Acrylamide is an important chemical used since the mid-1950s (Xie et al., 2006) and can be harmful to living organisms. LoPachin (2006) proved that it is neurotoxicant in both humans and laboratorial animals. This product is a vinyl monomer, produced by the hydration of acronitrile (Carere, 2006) and has multiple chemical and industrial applications. Besides those aspects, it is present in a series of fried, baked and heat-processed starchy foods (FAO/WHO, 2002), is used to produce water-soluble polyacrylamides, as a flocculant for clarifying drinking-water, as cosmetic additives and also in paper and paperboard food packaging and coating. Is also a component of tobacco smoke, which indicates that it can be formed by heating of biological matter (Carere, 2006). Accordingly, continuous exposure to small amounts of acrylamide is common. World Health Organization (WHO) published data of the average daily intake of acrylamide for the general population, as being 1μg/kg (Xie et al., 2006).

A consultation convened by FAO/WHO in 2002 recognized the presence of acrylamide in food "as a major concern in humans based on the ability to induce cancer and heritable mutation in laboratories animals". The European Commission (EC) Scientific Committee on Food (SCF) (Carere, 2006} reached a similar conclusion in 2002. Acrylamide is a simple compound; its chemistry is based in alpha and beta unsaturated double bonds. Its metabolic conversion leads to glycidamide, an epoxide metabolite very reactive with electrophiles (DNA), but a second important process occurs; it is called a Michael-type reaction, in which the beta-carbon reacts with a nucleophile (proteins). These two reactions are important because they explain the primary targets of acrylamide (proteins) and glycidamide (DNA) (Carere, 2006). One of the most important reaction of acrylamide with proteins is the adduction of hemoglobin (Hb). Adducts are formed at the SH groups and at the amino groups of the N-terminal valines (Xie et al., 2006).

It is important to remember that acrylamide is metabolized by P450 CYP2E1 to glycidamide, which is responsible for many toxic effects, especially genotoxic (Edwards, 1975; Sumner et al., 1992). Both acrylamide and glycidamide can form

adducts with sulfhydryl and amino groups of hemoglobin (Hb) (Carere, 2006) and other proteins. In this way the genotoxic effects of acrylamide and the metabolic glycidamide can explain many of results obtained with *D. melanogaster* exposed to acrylamide.

Acrylamide is oxidized to glycidamide, a reactive epoxide, and undergoes conjugation with glutathione. DNA adducts from glycidamide have been reported following administration of acrylamide (Dybing and Sanner, 2003). This chemical is capable, therefore, of interacting with vital cellular nucleophiles possessing –SH, $-NH_2$ or –OH (Yousef and El-Demerdash, 2006), including, consequently, side chains from residues of any protein.

Drosophila, it was recommended by the European Center for the Validation of Alternative Methods (ECVAM) as an appropriate model for research and as one of the best models for human diseases (Siddique et al., 2005). This holometabolous insect has a number of advantages for the detection of the genotoxicity of chemical substances (Würgler et al., 1984). These include ease of husbandry, rapid life cycle, small number of chromosomes, and ease of chromosome manipulation (Wilson, 2001).

The present work aimed to establish some behavioral, biochemical and morphological responses of *Drosophila melanogaster* exposed to ten generations of *D. melanogaster* to low concentrations of acrylamide (50 μM). Given *D. melanogaster* is a model insect for toxicological tests and the genetic and molecular biology of this species is already documented, the effects of acrylamide could be comparing to other organisms.

Materials and Methods

The experiments were carried out using *Drosophila melanogaster* collected by traps (Medeiros and Klaczko, 1999) from natural populations in São José do Rio Preto, State of São Paulo, in may 2005. The tests were performed using two groups: an isofemale strain homozigous for esterase 6 and a massal strain. The flies were maintained in culture chambers at constant temperature (25°C) and humidity on a banana/agar medium.

Acrylamide was added to the culture medium, at a final concentration of 50μM, equivalent to 3.31mg/g of banana/agar medium in 250 mL bottles. A control group was maintained without acrylamide addition. Experimental and control group were doing in three replicates.

The reproductive ability (productivity) was analyzed through 10 generations. Twelve virgin females and males recently emerged were collected from the isofemale strain in order to product the first generation; for the other generations, the same number of couples were picked up among the flies emerged on the fifth day after the first adults have emerged in the previous generation. So in each generation the female was allowed to lay eggs for ten days and the emerged adults were counted for nine consecutive days, followed by counting in the 11^{th}, 13^{th} and 15^{th} days. Emerged adults were analyzed morphologically (tergites' pattern and color, wings opening and shape, and eyes' color). This same procedure was carried out for the massal strain too, but only F_1, F_5 and F_{10} were analyzed.

Using the isofemale strain other experiments were carried out: the insects' weight was verified in F_1 and F_{10}. Adults emerged during three hours were scored and transferred to 1.5 mL tubes to be weighted in an analytical scale AND HR-200. It was done in a period of six consecutive days. For the purpose of egg-to-adult development analysis, three couples of virgin females and males were maintained separately in vials containing banana-agar medium for five days. In the 6^{th} day each couple was put in contact in bottles containing a spoon filled with agar/sugar medium, where the female laid the eggs. The eggs were counted and the spoon was transferred to a culture medium containing or not acrylamide (50μM). Pupae and adults were counted. This procedure was performed for F_3 and F_{10}.

In order to analyze the effect of different acrylamide concentrations (25, 50 and 100 μM), one female and one male from F_8, were allowed to couple for 24 hours in a vial with culture medium. The male was removed and the female was allowed to lay eggs for three days. Afterwards this female was transferred to a new tube containing the same culture medium for another 3 days, and subsequently was transferred again. This procedure was repeated seven times. The offspring of each female was counted. Three replicates were done for each acrylamide concentration and nine for the control.

We performed mating tests, observing pre-copulation and copulation times during one hour. Adults from F_1 and F_{10} were separated by sex in groups of five, for six days in tubes containing culture medium. Then, the females and males were transferred, without anesthesia, to a single vial, without culture medium to be analyzed the pre-copulation and copulation times using a digital chronometer, as performed by Itoyama (1992).

Statistical analysis for the experiments involving productivity was carried out using the 2-proportions equality test (Z-normal approximation) (Moore, 2005) and their graphs were represented as the proportion of emerged flies (observed/ total of generation) For the other experiments we applied the Student´s *t* test (Zar, 1999). Differences with $p<0.05$ were considered significant. Statistical analyses were performed using the BioEstat 4.0 program (Ayres et al., 2005).

Results

In the F_1, F_2, F_6, F_7 and F_{10} generations the average emergence in 50 μM acrylamide treated group was higher than in the control one (Figure 1), however, only for F_1 Student's *t* test revealed significant differences. In figure 2, the first contact with acrylamide (F_1), presented two emergence maxima compared with control group. In the following generations (F_2 to F_5) the emergence profile of the exposed group had similar trend of the control one. In F_6 (Figure 2) the control maximum emergence was in the 6th day, whereas for the treated group there was a delay: it occurred in the 7th and 8th days. The difference in 8th day was significant. In F_7 the first maximum emergence in the control group was in the 2nd day, and the second maximum was in 6th day for control group. For treated group 3rd, 5th and 9th days have the highest emergence proportion value in all the 7th generation, according to this, a delay in the emergence pattern was observed. The analysis of F_8 and F_{10}, especially, showed that in the last day (15th) the emergence tended to increase, whereas in the control group the trend was the opposite (Figure 2).

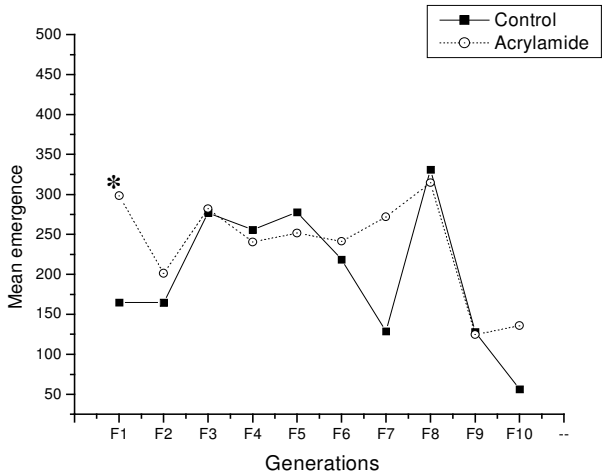

Figure 1 - Graph of mean emergence of 10 generations in the control and acrylamide exposed group. * indicates p<0.05.

A comparative study of the isofemale line (IL) and the massal line (ML), according to figure 3 showed that in the former the emergence decreased in F_{10} in both the control and exposed groups, but in the massal line the emergence increased (Figure 3). In the statistical analysis comparing the exposed and control groups of the massal line, in F_1 the emergence was significantly different.

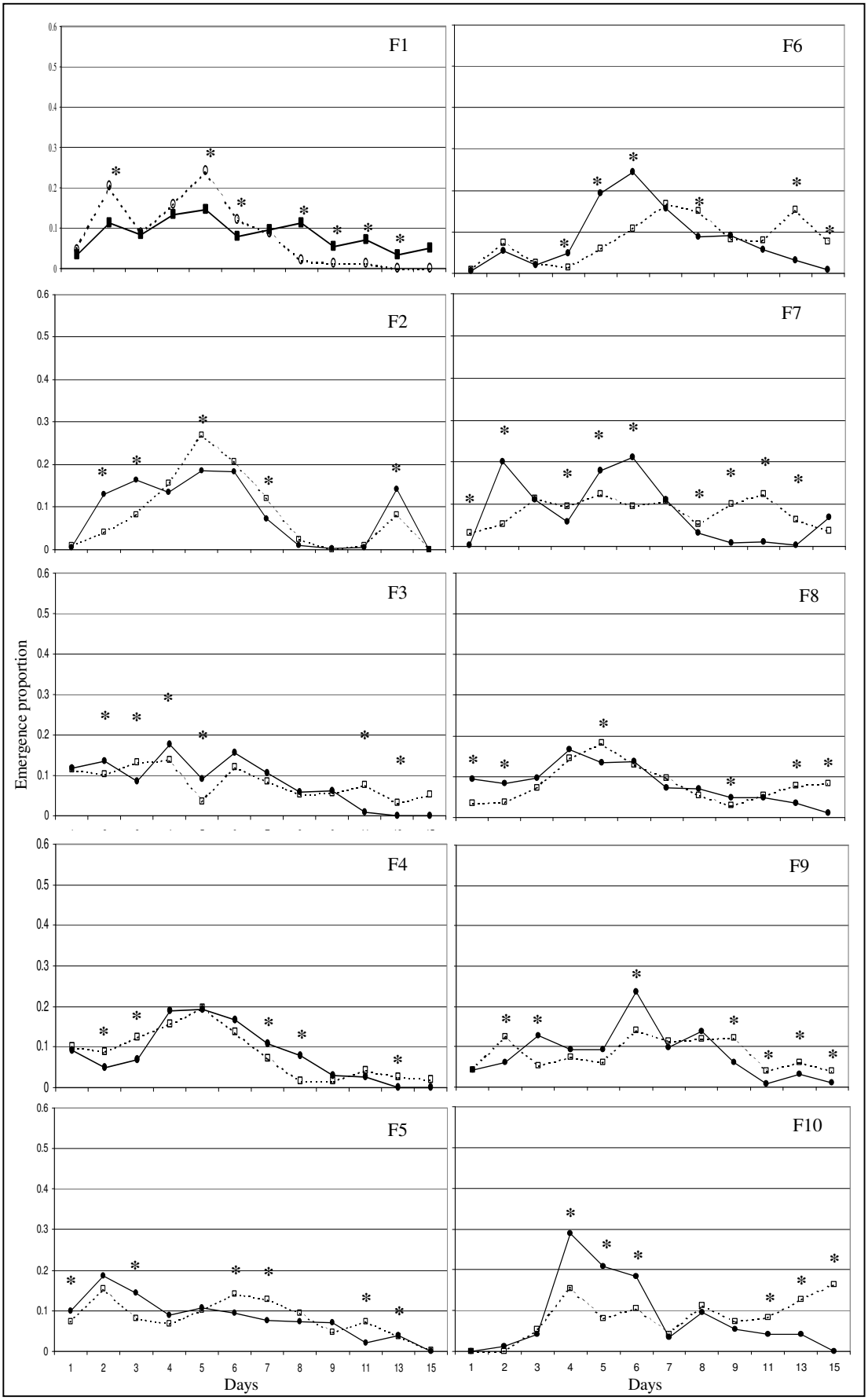

Figure 2 – Graphs of emergence proportion in 10 generations of *D. melanogaster* (F_1 to F_{10}), where continued line was control group and dashed line was the treated group with acrylamide. Asterisks indicates $p<0.05$

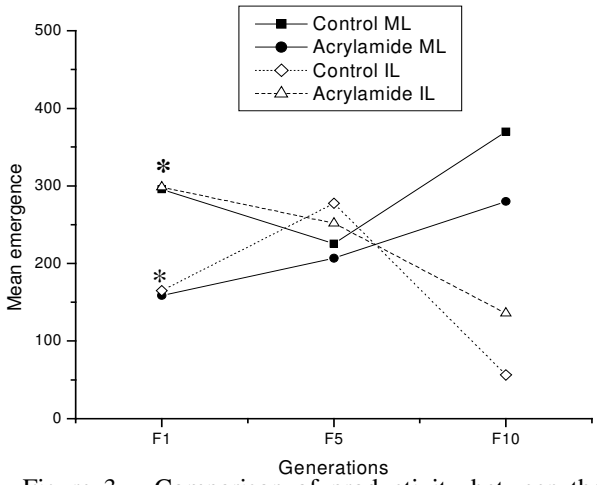

Figure 3 – Comparison of productivity between the massal and isofemale strains for F_1, F_5 and F_{10}.

In the isofemale line the percentage of adults with phenotypic alterations was greater in the treated group in most of the generations (F_1, F_3, F_4, F_5, F_6, F_7 and F_8), in figure 4. However, significant differences occurred only in F_6. Wing alterations were more frequent (in eight generations) than those affecting the tergites, that appeared in only five generations (Table 1 and Figure 4). It was observed one male with altered eye color in the group exposed to acrylamide (Figure 5).

The effect of acrylamide on the life cycle of *D. melanogaster* was investigated in F_3 and F_{10} generations. It was observed that the contact with the product reduced oviposition. In F_3 and F_{10} emerged viable adults from half of the eggs; this value in the control group was 97.9% in F_3 and 72.3% in F_{10}, as shown in table 2. Acrylamide exerted a greater effect on the transition from eggs to pupae, since about 55% of the eggs did not eclode in F_3 and F_{10}.

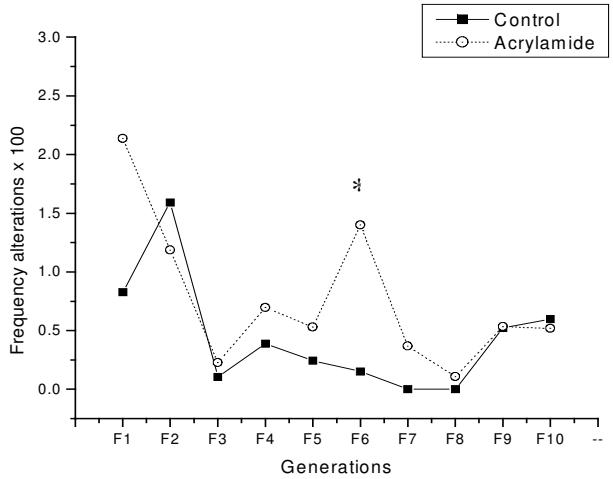

Figure 4: Percentage of alterations in 10 generations of the isofemale line from *D. melanogaster*.

Table 1: Percentage of wing and tergite alterations in each generation for the control and exposed to acrylamide groups from the isofemale line of *D. melanogaster*.

	Generations	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	F_9	F_{10}	Total
Wings	Control	0.83	0.79	0.11	0.00	0.24	0.00	0.00	0.00	0.26	0.00	2.23
	Acrylamide	2.14	0.99	0.11	0.56	0.00	0.15	0.37	0.11	0.27	0.52	5.22
Tergite	Control	0.00	0.79	0.00	0.39	0.00	0.15	0.00	0.00	0.26	0.60	2.19
	Acrylamide	0.22	0.00	0.11	0.53	1.24	0.00	0.00	0.00	0.27	0.00	2.37

Figure 5: Examples of morphological alterations in *D. melanogaster*: A- eye's color; B: tergite and C: wings

Table 2- Mean values and standard deviation in control and acrylamide treated groups concerning eggs, pupae and adults counting in F_3 and F_{10}.

Generations	F_3		F_{10}	
	Mean		Mean	
Stage/Product	Control	Treated	Control	Treated
Egg	47 ± 12.5	20.5 ± 6.4	21.5 ± 3.5	12 ± 5.6
Pupae	46 ± 9.5	11 ± 14.1	16.5 ± 0.7	7 ± 8.4
Adults	46 ± 9	11 ± 14.1	15.5 ± 2.1	6.5 ± 9.2
Viability Egg-adult (%)	95.5 ± 6	45.1 ± 54.9	72.3 ± 2	40.6 ± 57.4
Viability Egg-pupal (%)	96.1 ± 5.1	45.1 ± 54.9	71.6 ± 9.5	46.9 ± 48.6
Viability Pupal-adult (%)	99.0 ± 1.0	100 ± 0	93.8 ± 8.8	50 ± 70.7

Mating tests were carried out for F_1, F_5 and F_{10} (Figure 6 A) showed that the pre-copulation time increases in all the generations. For the copulation time it was observed a decreased only in F_1, but these differences were not significant.

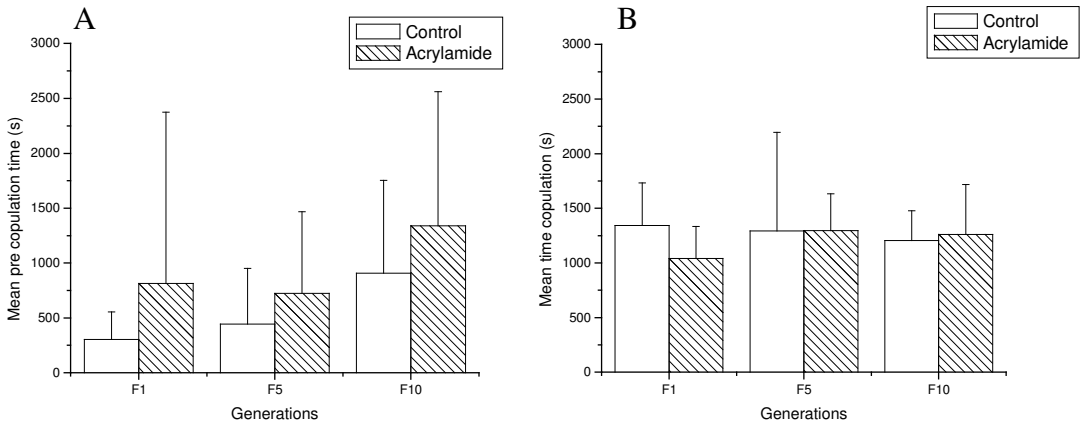

Figure 6: Graphs of A: mean pre copulation time in F_1, F_5 and F_{10} and B: mean copulation time in F_1, F_5 and F_{10} for the control and acrylamide treated groups.

The influence of acrylamide was also analyzed in respect to the insects' weight. In F_1 the average weight decreased in the adults from the treated group, but in F_{10} flies' weight is similar for both groups (Figure 7). These differences were not significant.

The analysis of the effect of different acrylamide concentrations (25, 50 and 100 μM) on the emergence, showed that the lowest concentration induced a decrease, and as concentration increases, emergence raises accordingly, as showed in figure 8.

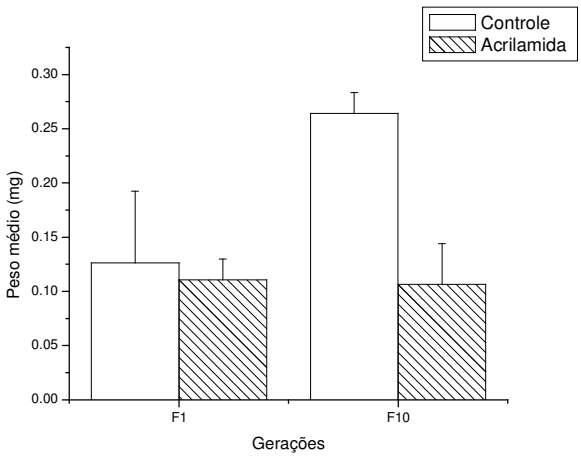

Figure 7- Graph of the mean weight of adults in F1 and F10 generations for control and treatment groups.

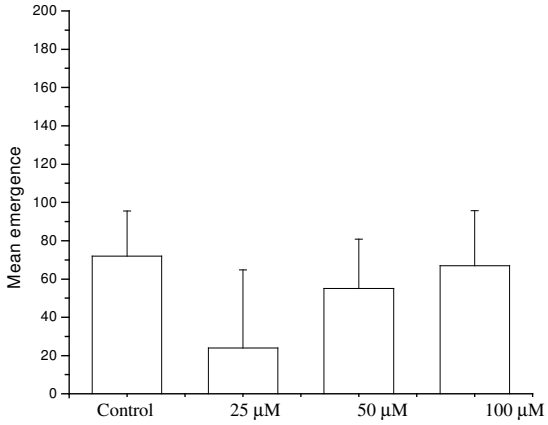

Figure 8- Adult mean emergence from F_8 flies submitted to different acrylamide concentrations.

Discussion

Acrylamide is a compound known to produce peripheral neuropathy in animals and man (Kuperman, 1958). Neurotoxic effects in humans are well known, based on occupational and accidental exposure. Treatment with this drug caused *in vivo* somatic mutations (Neuhäuser-Klaus and Schmahl, 1989), heritable translocation (Shelby et al., 1987) and specific locus mutations in mice (Rusell et al., 1991) and dominant lethal mutations in both mice and rats (Shelby et al., 1987; Working et al., 1987). Specifically, as cited by Trypathy et al. (1991), this product was non-mutagenic in Salmonella and *Klebsiella pneumoniae*, can induce chromosomal aberrations in mouse spermatogonia, and also dominant lethals in mice and rats. In *Drosophila*, after sex-linked recessive tests, this product seems to be mutagenic (Tripathy et al., 1991).

The emergence profile along ten generations of the isofemale strain (Figure 2) revealed two responses: first, delays of the maxima in the exposed groups (F_6 and F_7). A second observation is that in some generations there was a trend to continue the emergence after the 15th day; in other words, a delay in generations F_8 and F_{10}. In the first generation the proportion of emergence was higher in the treated group and this can be verified in F_7 and F_{10} too, but in the other generations, this value is similar for both groups (Figure 1). Another point is that only after five generations of continuous exposure, the emergence analysis showed some differences (Figure 2).

For a direct comparison between both *Drosophila* strains, we must focus in F1, F_5 and F_{10}. Effects of acrylamide were observed in F_1 and F_{10}, but in F_5, the emergences were similar (Figure 3). This difference can be explained in part by the peculiarities of each line, even though they were collected in the same region. Hirsch et al (2003)

postulated that *D. melanogaster* females can use different strategies facing a xenobiotic compound.

Considering malformations, along the ten generations of the isofemale strain of *D. melanogaster*, it was observed a trend in the group exposed to acrylamide. In F_6 the frequency of alterations was significantly higher in the treated group (Figs 4 and 5 and Table 1). According to Tripathy et al. (1991), acrylamide is genotoxic to the somatic and germ cells of *D. melanogaster* and can induce both single spots and twin spots in adult wings after larval exposure; this could explain the high percentage of alterations observed in some generations. Wing alterations were more frequent than those affecting tergites (Table 1).

We also performed an analysis of acrylamide effect on the biological cycle of the insect (Table 2). This product affected the eclosion and the emergence, and as mentioned above, acrylamide is genotoxic to the germ cells, and larvae exposed to concentrations ranging from 1.0 to 2.5mM (Tripathy et al., 1991). These results could explain the reduction of egg-adult eclosion.

Acrylamide has been also classified as an animal carcinogen and probable human carcinogen (Dearfield et al., 1988). Mice exposed to acrylamide had their sperm concentration and morphology affected (Sakamoto and Hashimoto, 1986), and in rats, morphology, motility and transport of sperm in uterus were affected too (Sublet et al., 1989; Yang et al., 2005), indicating that exposure to this product decreased matting frequency (Xie et al., 2006). Dearfield et al. (1988) suggested that acrylamide acts directly on the reproductive system rather than indirectly by causing stress and other systemic effects. It can be an explanation for the differences, especially in pre copulation time (Figure 6A). According to Odland et al. (1994), acrylamide is a well-known neurotoxic compound that produces central and peripheral distal axonopathy. Peripheral neuropathy and inhibition in the activity of acetilcholinesterase (AChE) (Yousef and El-Demerdash, 2006) are also detected in experimental animals and humans.

Another effect of acrylamide was the reduction of the average weight in the treated flies (Figure 7). Based on the literature, the treatment with acrylamide could cause a reduction in the levels of protein in plasma and different tissues, and the rate of protein synthesis could decrease in a concentration-dependent manner in response to acrylamide exposure (Odland et al., 1994). According to Chatterjea and Shinde (2002),

retardation of body weight gain observed in acrylamide-treated animals in comparison to control group indicates excessive break down of tissue proteins.

At different acrylamide concentration our results showed that there is an effect on flies emergence, as acrylamide concentration is increased. According to Yousef and El-Demerdash, 2006) experiments conducted with rats indicated that acrylamide caused disturbances in the oxidative status and enzymatic activities, and the effect was more pronounced with higher doses. An analogous behavior was observed in our experiments.

In summary, based on our study, acrylamide, although used in low concentration, can cause harmful effects as evaluated by a continuous exposure, and as demonstrated, when the concentration increases the effects tend to increase too. Damages were observed in terms of behavior (pre copulation time), some perturbations affected the life cycle and particularly the eggs, in viability of eggs.

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Effects of low concentrations of Lead, Chromium and Aluminum, on the productivity of *Drosophila melanogaster*

Este artigo será submetido para Toxicology Letters

Capítulo Quatro

Effects of low concentrations of Lead, Chromium and Aluminum on the productivity of *Drosophila melanogaster.*

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Abstract

Worldwide pollution by heavy metals has risen dramatically principally because of the industrialization process. Although certain metal ions are vital for many biological processes, their accumulation can be harmful. One of the metals that have a postulated biological function is chromium, although it is also genotoxic and probable carcinogenic too. Concerning inorganic lead, is a probable carcinogenic to humans, and together with aluminum these elements do not have any known physiological purpose. Aluminum seems to be related to neurodegenerative diseases. Resistance or tolerance to certain products is evident in invertebrates. For research with metals *Drosophila* is a convenient system since many aspects of metal homeostasis are conserved between flies and human. This study followed up ten generations of *Drosophila melanogaster* exposed to chromium, lead and aluminum. For comparison purpose, two stocks of *D. melanogaster* were used. Results indicate that for each metal, the insects respond in a different way, with differences in the amount of emerged flies and the day(s) when productivity reaches a maximum value. All the metals affected also the morphology, leading to an increased number of malformations in tergites, wings and coloration pattern.

Keywords: biomonitoring, heavy metals, lead, aluminum, chromium, *Drosophila melanogaster*, productivity, bioaccumulation.

Introduction

Pollution has become one of the most important modern concerns, since many of its impacts affect our world. A trend in ecotoxicology and ecological risk assessment is to develop new methods for monitoring populations stressed by chemical pollutants (Graham et al., 1993). In the history of toxicological studies, Valentine and Soulé (1973) suggested that developmental instability might be a stress indicator.

Heavy metal pollution has risen dramatically in the last 50 years as a result of an exponential increase in their use in manufacturing products. The evolution of heavy metal tolerance has been well documented in natural populations of hundreds of species around the world (Antonovics et al., 1971; Rosenheim et al., 1996). Heavy (or toxic) metals are trace elements that are at least five times denser than water. They are stable elements (cannot be metabolized by the body) and bioaccumulative (e.g.: mercury, nickel, lead, arsenic, cadmium, aluminum and chromium). Much of damage produced by toxic metals arises from the proliferation of oxidative radicals they cause, O_2^-, H_2O_2 and OH^-.

Some metal ions are vital for many biological processes, such as transcription, respiration and growth. However, over accumulation of essential metals or even non-essential toxic metals is injurious (Yepiskoposyan et al., 2006). One of the metals that have biological function is chromium, proposed to participate in glucose's and lipid´s metabolism (Sano et al., 2000). Its accumulation can cause severe damage to the body, but the toxicological effects depend on the ionization state, Cr(III), Cr(IV), Cr(V) or Cr(VI). Cr(VI) is much more toxic than Cr(III) (De Flora et al., 1987 and Suzuki, 1988). This fact has been explained because cells, via anion carrier proteins, easily take up Cr(VI) (IARC, 1990). Under physiological conditions Cr(VI) does not seems to react with DNA, although once inside the cells it is reduced by H_2O_2, glutathione reductase, carbohydrates, ascorbic acid, and other molecules to more reactive products, including Cr(V) and Cr(IV) and the most stable form, Cr(III). This reduction process causes the generation of active oxygen species (Kawanishi et al., 1986). The genotoxicity effects of chromium compounds have been extensively studied using different test systems and measuring various targets and/or genetic endpoints (Amrani et al., 1999).

Another metal derivated from pollution processes is lead, also widespread in the environment. Most of it comes from human activities like mining, manufacturing and burning of fossil fuels. According to the International Agency for Research of Cancer, inorganic forms of lead are probable carcinogenic to humans.

About 10% of this metal can be absorbed by the digestive system (Corey and Galvão, 1989). If administered in excess it can compete with calcium (Pounds, 1984) and can inhibit heme group synthesis (Castañeda et al., 2001). In children, lead can affected the behavioral development (Hirsch et al., 2003).

A third metal, aluminum, is among the most abundant elements on Earth. This element is found in small amounts in all organisms, but no functional purpose has been reported (Williams, 1999). Aluminum is specially found in corn, yellow cheese, salt, herbs, spices, tea, cosmetics, aluminum ware and containers. It is also present in medicines and added to drinking water for purification purposes (Ochmanski and Barabasz, 2000).

Resistance or tolerance for certain products is evident in invertebrates (Kalajdzic *et al*., 2006). Insects respond to toxins (metal ions and insecticides) involving biochemical and genetic mechanisms (Wilson, 2001). In particular, *Drosophila melanogaster* is a widespread species, commonly used in genetic studies. It requires inexpensive culture media, has short generation time (about 10 days at 25ºC), raises a large number of individuals per generation and assays *in vivo* are easily feasible (Wilson, 2001). For research with metals, specifically, *Drosophila* is a convenient system to address questions of contamination, since many aspects of metal homeostasis are conserved between flies and human. In recent years numerous studies led to a better understanding of uptake, distribution, detoxification and elimination of metal ions (Yepiskoposyan et al., 2006).

This study describes some effects of three metals' bioaccumulation: chromium, lead and aluminum, in two stocks of the bioindicator, *Drosophila melanogaster*. The data were gathered along ten generations, with daily scored emergence of the adults. The morphological alterations were also scored for each product.

Materials and methods

Chemicals

Aluminum chloride, potassium dichromate and lead nitrate were used in a final concentration of 50µM, dissolved in distilled water and added to the banana-agar culture medium (banana, agar, water, sugar and nipagin as fungicide). In order to avoid the change of oxidative state of the chromium, the mixture was prepared under controlled temperature, at approximately 45ºC. Medium culture (30 mL) were transferred to 250 mL glass bottles and used to feed the fruit flies.

Strains

Specimens of *Drosophila melanogaster* were collected in May 2005 at São José do Rio Preto, State São Paulo, Brazil. Two stocks were created: an isofemale (IL) and a massal strain (ML). This strain was formed by five couples. The insects and the experimental cultures were maintained in a temperature-controlled chamber at 25 ± 1ºC, feeding from a banana-agar culture medium.

Generation analysis

For each experiment the flies were maintained in a contaminated (test groups) or ordinary medium culture (control group) for ten generations. From the first generation, 12 couples recently emerged were removed from the isofemale or massal stock. For each metal and for the control (without metals) we did three replicates. After five days of the first emergence, 12 new couples were taken away to start a new generation, and this whole procedure was repeated for the other generations. The parental couples were allowed to stay in 250 mL glass bottles for ten days and for the isofemale strain the offspring was scored for 9 consecutive days and after at the 11^{th}, 13^{th} and 15^{th} days. The adults were analyzed morphologically (tergites color and pattern, wings shape and opening, eye's color, body pigmentation). For the massal strain, these analyses occurred only in F_1, F_5 and F_{10}.

Statistical analyses

Statistical significance for the productivity experiment was determined using the 2-proportions equality test (Z-normal approximation) (Moore, 2005). The differences between the test groups and their controls were analyzed by Student´s *t* test (Zar, 1999). Differences with $p<0.05$ were considered significant. Statistical analyses were carried out using the program BioEstat 4.0 (Ayres *et al*., 2005).

Results

Concerning the isofemale strain, in daily analyses for aluminum (Figure 1) the first three generations showed a similar curve pattern for test and control groups in terms of proportion. In the other three generations (F_4 and F5), the first day of emergence for the test group was one of the highest of the generation and was always superior to the emergence in the control. In F_7 both the control and the test groups has two emergence maxima, but for the treated group the peaks happened one to two days

after the control. For the 9th generation, the emergence presented the maximum in the first day, like in F_4 and F_5, and in the following days the curve were similar to their controls, but in a lower proportion. In the last generation, the exposed group had two maxima: one was in the 3rd day and the other in the 6th day. Between both days occurred the control maximum (4th day). Six of the profiles showed delayed emergence in the last days of the 15 days period.

In the presence of chromium (Figure 2) the effects were different from aluminum. Seven of the ten profiles displayed a higher proportion of emergence for the test group at the end of the counting period, some of them suggesting strongly (F_1, F_3, F_9) that it would continue beyond the 15th day. For F_{10} the maximum of emergence in the control was in the 4th day, but in the treated group was in 6th day, a sharp maximum, with a higher proportion than in the control. On the other hand the generation finished in 15th day, and the trend to continue was not observed.

For lead (Figure 3), we can see that six of the ten profiles showed, also for this metal, a delay of their emergence. That is particularly clear for F_1, since the test group score at the 15th day presented a higher proportion than the control, and it was observed in F_3 too. For the second generation, the differences involving the emergence maxima between the control and the treated group were in the 4th and 5th days. In these days occurred the emergence maximum of the treated group, not corresponding to the control maximum. These days were exactly in the middle of the emergence highest points for the control. F_6, F_7 and F_{10} also presented clear differences from the control. For example, in F_7, the 4th day of emergence for the test group was in the middle of the two days of maximum emergence (2nd and 6th days) of the control. In F_{10}, as occurred with aluminum and chromium, the maximum day of emergence for the treated group (6th) had a delay of two days after the control (4th), and for the second maximum, the difference was one day (8th day for control and 9th day for the treated group).

An analysis of the mean emergence per generation (Figure 4), showed for metals a high value for F_8, and a clear decrease for F_9 and F_{10}. The groups exposed to chromium and lead also had emergence maxima in F_1 and F_5. On the other side, the replicates exposed to aluminum had secondary peaks at F_3 and F_6. Globally, the exposed groups showed from five to seven generations with emergence mean values above those from the control. Concerning the general shape of the profiles, for the groups exposed to lead and aluminum the values follow more or less closely those from the control. For the animals treated with chromium (Figure 4B) the generations F_1, F_5, F_7 and F_8,

presented higher mean emergence than the control (in F_7 the mean emergence for the treated was about 67% higher than the control one). For all the experimental sets the last generation had the lowest mean value for emergence, and its also occurred in the control.

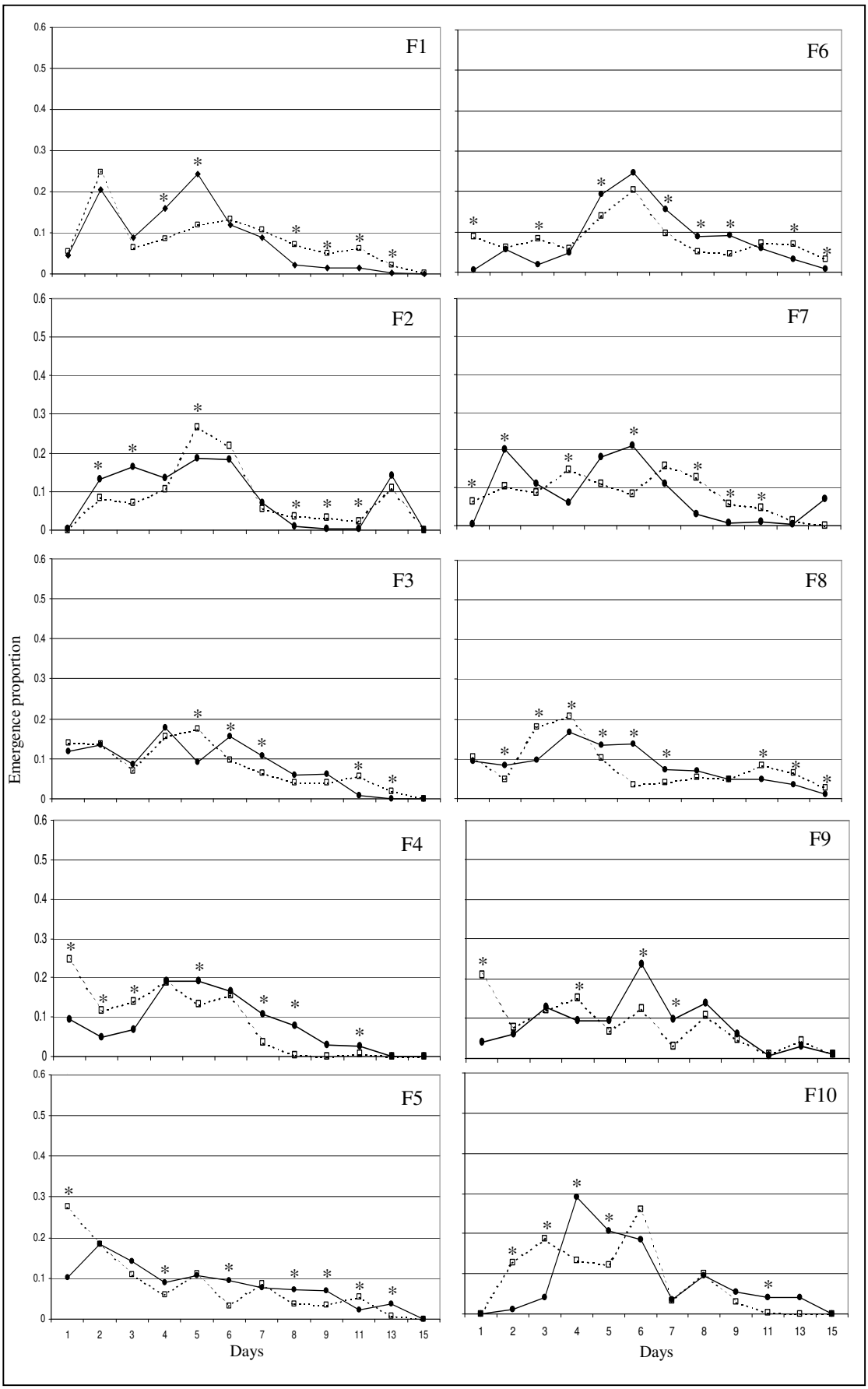

Figure 1 – Emergence proportion along 10 generations of *D. melanogaster* exposed to 50 µM of aluminum (isofemale strain). *C*ontinuous line: control group, dashed line: test group. The asterisks indicates $p<0.05$

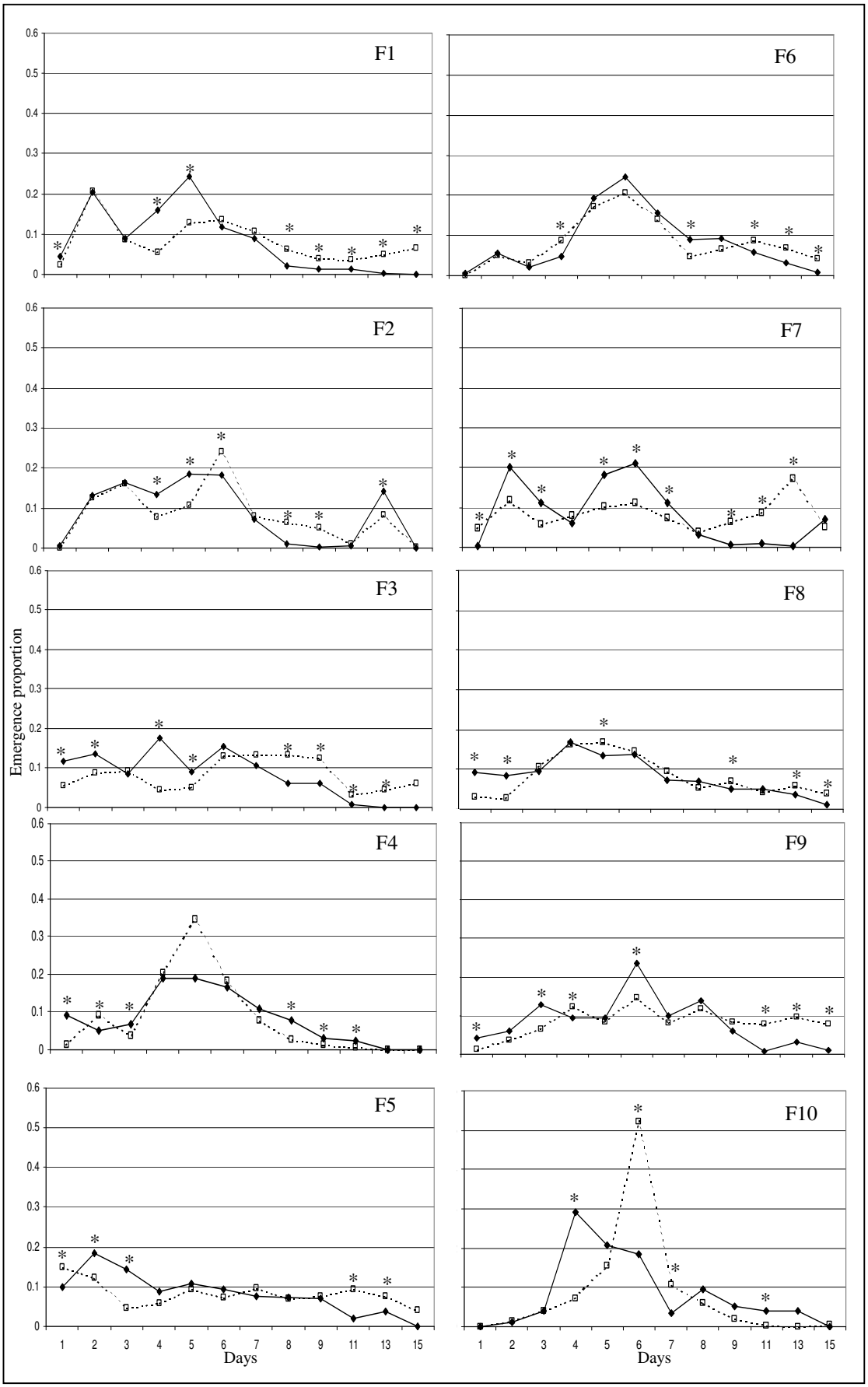

Figure 2 – Emergence proportion along 10 generations of *D. melanogaster* exposed to 50 µM of chromium (isofemale strain). *C*ontinuous line: control group, dashed line: test group. The asterisks indicates $p<0.05$

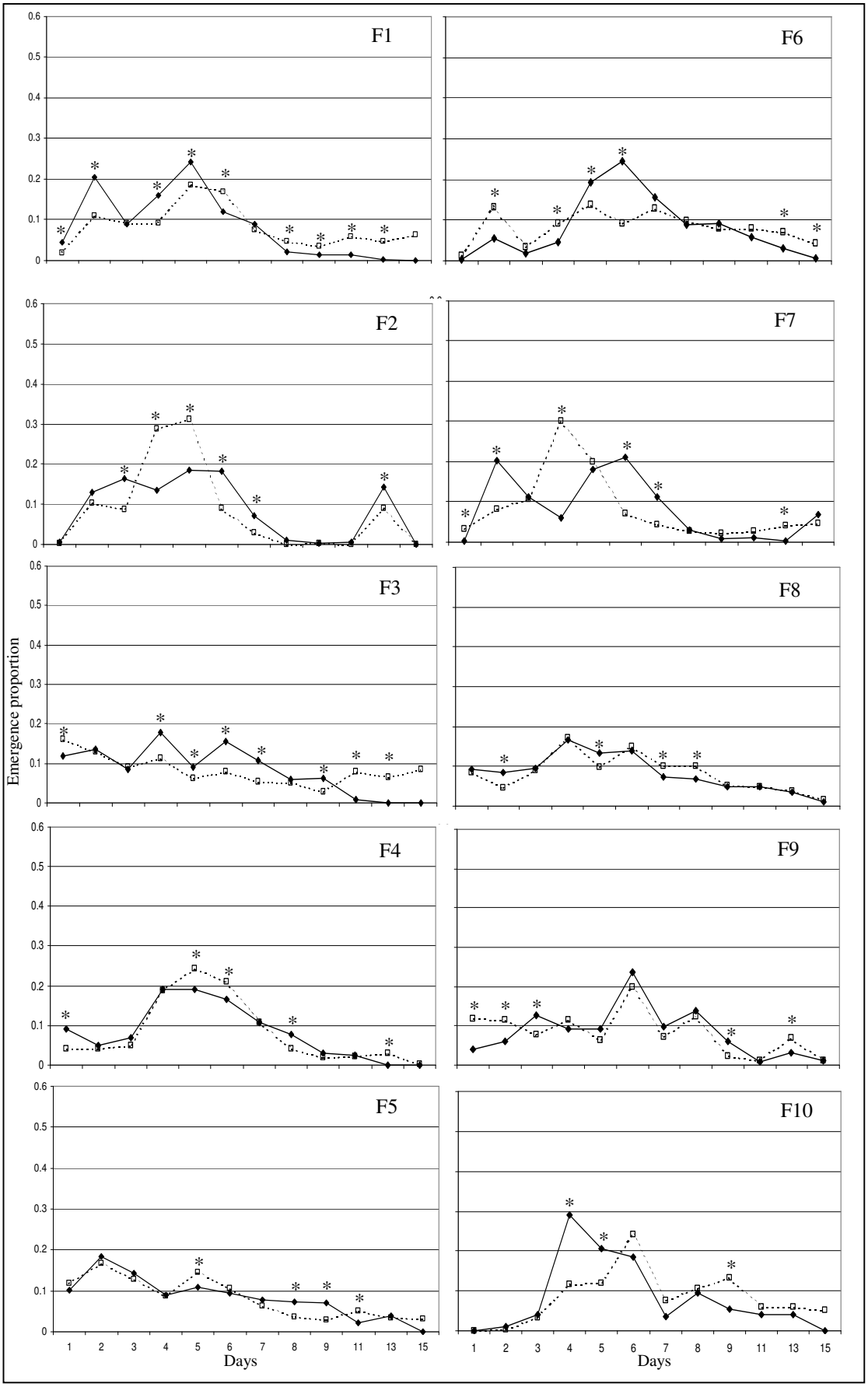

Figure 3 – Emergence proportion along 10 generations of *D. melanogaster* exposed to 50 µM of lead (isofemale strain). *C*ontinuous line: control group, dashed line: test group. The asterisks indicates $p<0.05$

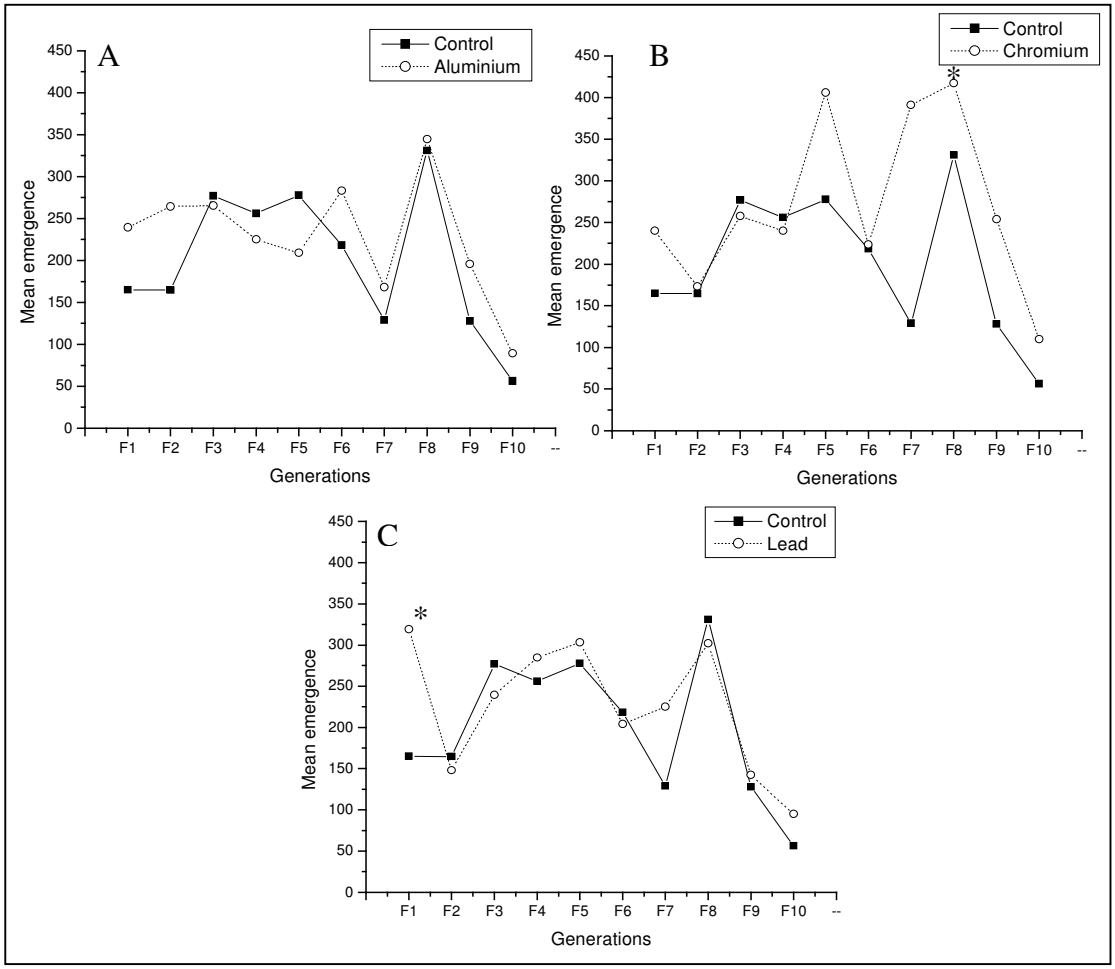

Figure 4: Graphs of average emergence. A: control and aluminum chloride; B: control and potassium dichromate; C: control and lead nitrate. The asterisks indicate that $p<0.05$.

During the fifteen days analyzed, simultaneously with the emergence scoring, it was evaluated and quantified the morphological for wings, tergites and other evident malformations, in each generation for each metal. The values were represented in terms of frequency percentage (Figure 5A). Looking at the plots, all the metals displayed a higher frequency in the first and last generations, although it is not so evident for lead. This observation is equally valid for the control, so appears that the effect of the metals, even reaching in many generations a higher frequency of malformations, followed a tendency present also in the non-exposed animals. These differences were not significant, although in some generations and for some products they were greater than for the control group. Aluminum was the metal that induced more morphological alterations, compared to lead and chromium. As observed in Table 1, for the exposure to aluminum, alterations involving the tergites were more frequent, and for lead, wing alterations (Figure 6A). All the metals affected the wings morphology significantly, specially lead. On the other side, the tergites did not display a significant frequency of alterations in the presence of this metal; in fact, the frequency was only about ¼ of that

from the control. Interestingly, chromium did not induce significant alterations of the wings or tergites. It is interesting to note that chromium and lead induced atypical alterations in the eyes' formation. In the presence of chromium, the right eye was not correctly formed, and instead of the common red coloration, they were yellow (Figure 6B). Another malformation involved lead: the left eye was not formed and in its place appeared a different structure, resembling an antenna (Figure 6C).

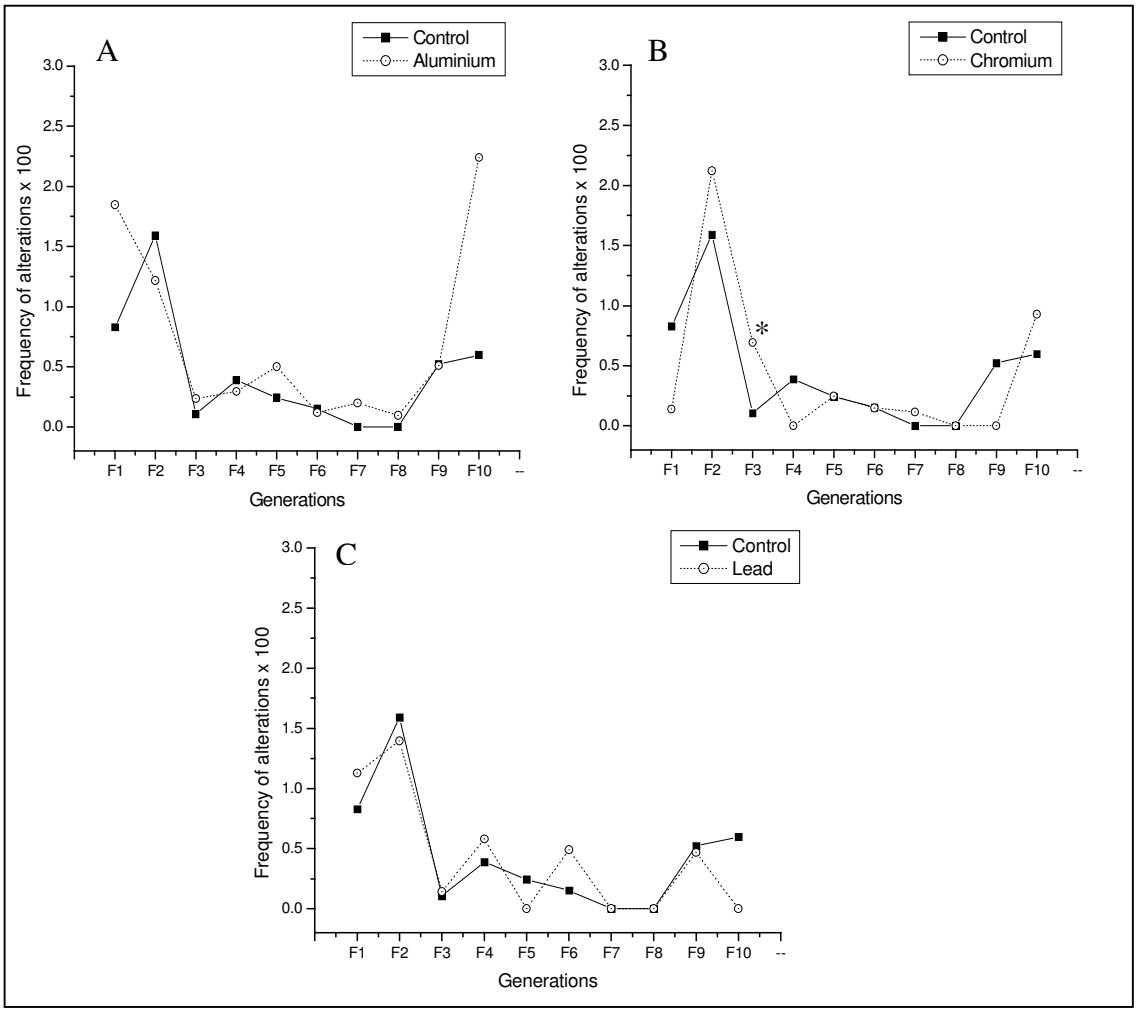

Figure 5: Malformation frequency for treatments A: aluminum; B: chromium and C: lead. * - indicates $p<0.05$.

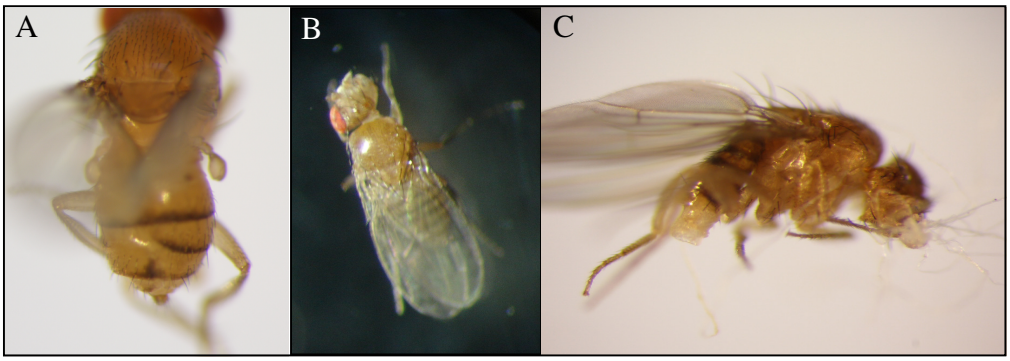

Figure 6: Examples of malformations affecting *D. melanogaster* exposed to metals: A: tergite alteration (Al); B: right eye alteration (Cr) and C: left eye alteration (Pb).

Table 1: Proportions (%) of wing and tergite alterations for *Drosophila melanogaster* exposed to metals, along 10 generations.

Alterations	Wings				Tergites			
Generations	Control	Aluminum	Chromium	Lead	Control	Aluminum	Chromium	Lead
F1	0.828	1.705	0	1.129	0	0.142	0.139	0
F2	0.796	0.456	1.179	1.397	0.796	0.608	0.943	0
F3	0.106	0.237	0.577	0.141	0	0	0.115	0
F4	0	0.148	0	0.463	0.389	0.148	0	0
F5	0.244	0.167	0.082	0	0	0.334	0.165	0
F6	0	0	0	0	0.152	0.118	0.149	0.490
F7	0	0	0.115	0	0	0.198	0	0
F8	0	0	0	0	0	0	0	0
F9	0.261	0	0	0.468	0.261	0.511	0	0
F10	0	0	0.310	0	0.599	2.239	0.619	0
Total	2.236	2.713	2.263	3.599	2.197	4.299	2.131	0.490

Another part of the study made a comparative analysis of the data described for two stocks: an isofemale (IL) and a massal (ML) line from *D. melanogaster*. The data showed differences for both stocks. Massal line: F_1 and F_{10} showed, for aluminum and chromium, lower emergence for the exposed groups. For aluminum at F_5 there was no difference of productivity between the exposed and control groups, but for chromium again the exposed sets had lower emergence. Lead had a different effect: the exposed groups had higher productivity than the control at F_1 and F_5, and a lower one at F_{10}.

Isofemale strain: For the three metals, the exposed groups had higher productivity than their respective controls for F_1 and F_{10}. The fifth generation showed higher mean values for the groups exposed to chromium and lead, whereas for aluminum the control was more productive.

Comparing now both strains, the greatest difference happened for F_{10}; the massal strain displayed, for the three metals, higher mean emergence values, but the control groups had even larger differences, maintaining the same tendency.

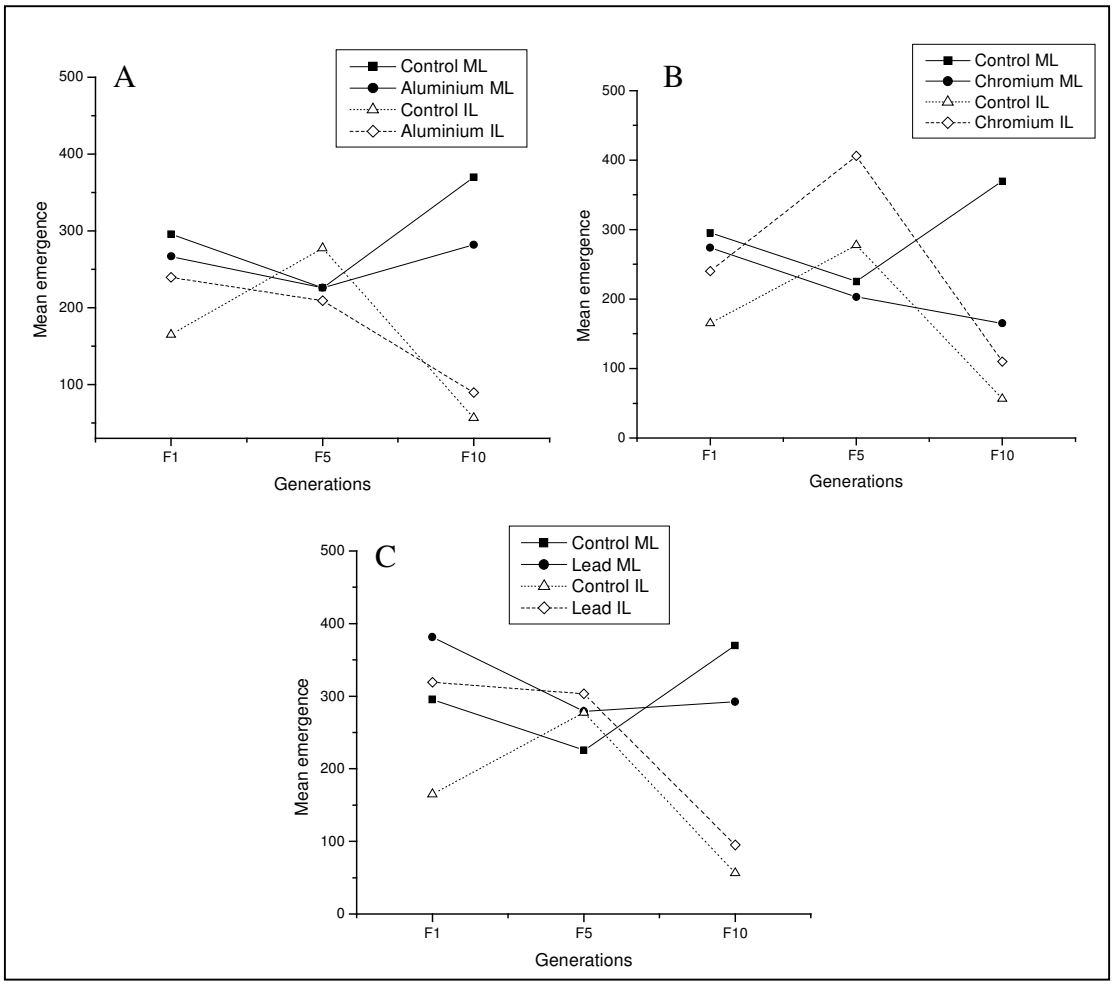

Figure 7: Comparison of the mean emergence of the isofemale (IL) and massal (ML) strains of *Drosophila melanogaster* in three generations for three metals: A: aluminum; B: chromium and C: lead.

Discussion

The results indicate that for each metal ion utilized in this work, the insects had diverse responses. It could be explained by the differences in the molecular mass or in the valence of each ion. For example, aluminum is a trivalent ion, but chromium is a hexavalent one and lead is a divalent. In this way each metal could be absorbed in a distinct manner, and compete with other cations for binding to enzymes, for example (Jacobson et al., 1983).

Aluminum

This is a metal ion known to be a pro-oxidant and has recently been implicated in the generation of an oxidative milieu by aiding in the production of reactive oxygen species (ROS) such as O_2^- and H_2O_2 (Mailloux and Appanna, 2006). In the daily analyses (Figure 1), only after three generations the curve of the treated group

demonstrated some differences from the control one. Some generations presented a trend to continue in the 15th day and others had the emergence maximum dislocated from the "peak" of the control.

Aluminum salts according to Yousef (2004), may bind DNA and RNA, inhibit enzymes like hexokinase, acid and alkaline phosphatases, phosphodiesterase and phosphooxidase. This toxicity comes from substitution of Fe or Mg ions, inducing disturbances in intracellular signaling, excretory functions and cellular growth. It may substitute some metals such as iron and calcium and consequently could help to destabilize biological homeostasis (Middaugh et al., 2006); in this way, these effects could explain the differences in the generation's emergence.

The last generation (F_{10}) was the most affected by aluminum; it presented the maximum dislocated one day before, and a second maximum after the control (Figure 1). According to the literature, aluminum concentrations in the whole organism increases during development and aging of *D. melanogaster*. Aluminum decreased the life span by as much as 20%, for 0.1mM of aluminum chloride and for 1mM of aluminum nitrate and sulfate (Massie et al., 1985). Another observation in F_{10} is the major frequency value for morphological alterations (Figure 5A).

One of the main manifestations of aluminum toxicity is lipid peroxidation and oxidative damage to biomolecules, such as lipids, DNA and proteins (Yousef, 2004); accordingly, it can alter physiological and biochemical characteristics of biological systems (Prakash and Rao, 1995). For human beings, there is a consensus that some aluminum is retained in the body, probably in the skeleton and some deposits in brain, but most of it is excreted in the urine within a few days or weeks, and the gastrointestinal tract provides an effective barrier to aluminum uptake (Priest, 2004), and in vertebrates the toxicity of aluminum influences the skeletal system, as a result of diminished resistance and increased tendency to bone breaking. It comes from lower collagen synthesis and slowing down of mineralization process (Ochmanski and Barabasz, 2000).

Chromium

The carcinogenicity and genotoxicity of chromium is related to the oxidative state and solubilities of the ions. De Flora et al. (1990) and IARC (1990) reviewed the carcinogenic and mutagenic activity of hexavalent salts, but in general the genotoxicity of the different chromium compounds can be attributed to the chromate ion, which

predominates at physiological pH, being capable to permeate cells membranes (Levis and Bianchi, 1982).

The profile of emergence (Figure 2) showed that in F_1 and F_3 in particular, in the last day scored (15^{th}) the proportion of emerged flies in the treated group increased. In F_7 and F_{10}, the maximum emergence happened four and one days respectively dislocated from the control. According to literature, flies induced by Cr(VI) had delayed larval development (Frei and Würgler, 1996). In F_5, F_7, F_8, F_9 and F_{10} (Figure 4B), the mean number of flies in the treated group was higher than in the control group. Some works affirm that chromium compounds significantly reduce fertility and development of experimental animals (Zivanov-Curlis et al., 2006). However, in this case, the strategy to survive in that adverse environment could be different; the increase in fertility could be a response to the presence of metal ion in order to increase variability of the offspring, and produce viable descendants resistant to that contaminant, as proposed by Hirsch et al., 2003. Another literature information to be considered is the toxicity activity of chromium; the ability to attack DNA depends on the redox cellular system, so in this case, the chromium was not reduced to chromate (CrVI) and was incapable to attack the DNA, which could increase the offspring mean.

Concerning the malformation frequency (Figure 5B), only in three generations this value was higher than in the control group: F_2, F_3 and F_{10}. According to Graf et al., 1992, in somatic cells of *D. melanogaster* the high genotoxic activity of hexavalent chromium oxide is over 90% due to recombinogenic activity, which is not influenced by cytochrome P-450-dependent xenobiotic metabolism and Hepburn et al., (2003) revealed strong somatic recombinogenic activity in the wings of adults of *D. melanogaster* that had been fed with Cr(VI) salts during the larval phase. It is important to consider that alterations appeared in all the generations, but in only three of them, this value was higher than in the control, but only in F3 the statistical analysis demonstrated $p<0.05$.

It is interesting to observe the fly with the right eye malformed (Figure 6B); the reactive Cr(VI) would cause different lesions in the imaginal disk cells of *D. melanogaster*, giving rise to mutational and/or recombination events. However this damage can be repaired using information of the homologous chromosome (Amrani et al., 1999). In spite of the high recombinogenic activity, in mitotic cells little activity occurs, but the damage in chromosome by the presence of chromium, can result in increase of mitotic recombination with the sisters chromatides. This results in erroneous

DNA paring. However, in the case of the imaginal disc cell, this occurs in larval stage that can sustain damages to the DNA, and still can be a viable adult (Hepburn et al., 2003).

Lead

According to Hirsch et al. (2003); *Drosophila* bioaccumulates lead, and that exposure to this toxicant can have significant effects on the number of females mating. The analysis of lead nitrate effects in *D. melanogaster* revealed that for treated group, in F_1, F_4, F_5, F_7 and F_{10} the mean number of emergents was higher than in the control (Figure 4C), but only in F_1 this difference was significant. Lead has been reported to reduce fecundity in *Drosophila melanogaster*, when 0.05 to 5.0% of lead acetate was added to the medium culture (Uysal and Bahceci, 1996; Cohn et al., 1992). Hirsch et al. (2003) also demonstrated that lead reduced the fecundity in *Drosophila melanogaster* with 2 μg/g and unaffected by exposure to 20 μg/g lead. The profile of emergents analyzed daily (Figure 3), showed that delays in the biological cycle were evident in some generations such as F_2, F_7 and F_{10}. The toxicity of lead in *Drosophila* is known to affect the time course of pre-adult development (Akins et al., 1992). The frequency of alterations in the treated group was higher in some generations: F_1, F_4 and F_6 (Figure 5C). Specimens of *Drosophila subobscura* when exposed to lead in high and low concentrations, had a change of chromosomal gene arrangements frequencies in the generations F_4 and F_6 (Kalajdzic et al., 2006), in accordance with the proportion values of morphological alterations.

Stocks' comparison

In the analysis of the two stocks used in this experiments, the response to the metals was different in each strain. In the isofemale strain (IL) for the three metals (Figure 7A, B and C) in F_1 and F_{10}, the mean emergence was higher than in their respective controls. As discussed by Hirsch et al., (2003), it could be a female strategy to survive in that adverse environment. The female may increase the times that she mates and/or the number of laid eggs, increasing genetic diversity of her offspring and maximizing the possibility that some of them could be well-adapted to the metal presence. It also possible that rather than exerting control, the female is losing it, mating sooner and more indiscriminately and laying larger quantities of substandard eggs (Hirsch et al., 2003)

It is interesting to note that for all the replicates from the isofemale line, in the last generation the mean number of emergents decreased, even in the control. It could be explained in a similar way as above; the number of flies decreased after ten generations of constant inbreeding, due to a genetic diversity decrease. This effect was not observed in the other strain (ML), where the variability was higher than in the IL strain, formed only with one female.

In the massal strain (ML) for aluminum (Figure 7A) and chromium (Figure 7B) the mean emergence for this group was always below the value for the control group. Another strategy used by insects, according to Hirsch et al. (2003) would be that females living in adverse environments may postpone oviposition until the environment improves. However, for the groups exposed to chromium (Figure 7B) the trend observed was a progressive decrease of the emergence with the generations. This was not verified for the groups exposed to aluminum (Figure 7A) where the profile of the control curve and the treated one, had the same pattern.

The study of Kalajdizic et al. (2006) with gene arrangements induced by lead showed that the genetic mechanism of heavy metal resistance in *Drosophila* is a highly complex and dynamic system, and could be linked with adaptive processes in the course of evolving a genetic response to heavy metals. Raes et al. (1992) and Van Straalen et al. (1987) affirmed that invertebrates have some mechanisms that could block the absorption of heavy metals in the gut. Alternatively, it is possible that one-day-old flies may not consume the media as readily as older flies and thus would be less affected (Jacobson et al., 1983). In this work, the flies were maintained in contact with the product only for one day, because they were scored daily. The presence of metal ion in the fly culture medium could induce changes in biochemistry, cellular biology, nervous system, in the genetics and other biological systems. Consequently, the result of possible damages could produce synergistic or antagonistic effects; in other words; could produce changes for adaptation in that adverse environment or not, or it could even mask toxic effects.

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Effects of low concentrations of Aluminum, Chromium and Lead on Behavior, Weight, Egg Viability and Carboxylesterase activity of an isofemale stock of *Drosophila melanogaster*

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Capítulo Cinco

Effects of low concentrations of Aluminum, Chromium and Lead on Behavior, Weight, Egg Viability and Carboxylesterase activity of an isofemale stock of *Drosophila melanogaster*

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Abstract

Exposure to environmental contaminants, including heavy metals, continues to be a widespread problem in many countries. In toxicological research and testing there has been a worldwide effort to reduce the use of vertebrates. In order to analyze the toxic activity of chromium, aluminum and lead, this study used them at a low concentration, in order to analyze the accumulative effects in some biological aspects of *Drosophila melanogaster*, including carboxylesterase activity, sexual behavior, flies' weight and egg viability. This work tested the effects of aluminum, chromium and lead. mixed to the banana-agar culture medium at a final 50 μM concentration. Egg viability was tested in two generations (F_3 and F_{10}). Chromium increased mean pre copulation time in F_1 and F_5, whereas in F_{10} copulation time was significantly increased too. Lead was also the metal that produced the greatest decrease in egg viability, to about 50% in F_{10}. The data showed that the main target of the metals here tested was the step from egg to pupa, since the transformation from pupae to adults was less affected. On the other side, aluminum increased the activity of carboxylesterase, and concerning the insects' weight, in F_5 it increased.

Keywords: *Drosophila melanogaster*, aluminum, chromium, lead, viability, emergence

Introduction

Rapid industrialization in developed and developing countries led to a substantial increase in the generation of industrial solid wastes (Siddique et al., 2005), and many are hazardous because they contain heavy metals (Seco et al., 2003). Some metals ions that can act as poisons in the organism; generally those with atomic mass higher than 190 have no essential function (Jacobson et al., 1983), such as lead, mercury, aluminum and cadmium. Other metals are essential, but above physiological concentrations they cause harmful effects (Yepiskoposyan et al., 2006 and Middaugh et al., 2006). Whether supporting essential functions or interfering with them, the manner by which a metal ion causes its effects must reflect its properties and the properties of the biological molecules with which it interacts (Jacobson et al., 1983). Heavy metals are a well-established cause of severe illness, and these concerns need to be addressed (Brodkin et al., 2007).

In the last years there is a worldwide effort to substitute vertebrates in toxicological research and testing, by invertebrates such as *Drosophila melanogaster*. This species has easy husbandry and chromosome manipulation, rapid life cycle, and small number of chromosomes, easy of genetic transformation and availability of its sequential genome (Wilson, 2001). After sequencing of the *Drosophila* genome, the similarities *Drosophila*-human have been strengthened, since 60% of human disease genes have homologues in this fly (Schneider, 2000). The similarities between *Drosophila* and higher organisms at the basic biochemical level of cellular function are well known (Jacobson et al., 1983).

Since metals are not metabolized, the opportunities in influencing toxicity are more restricted than for many organic chemicals, for which metabolic pathways may profoundly influence an organism's response (Gochfeld, 1997). For research with metals, the fruit fly is a convenient system to address contamination matters, since many aspects of metal homeostasis are conserved between flies and human. In the recent years numerous studies led to a better understanding of uptake, distribution, detoxification and elimination of metal ions (Yepiskoposyan et al., 2006).

One of the metals constantly present in the environment is chromium. It is ubiquitous in nature; can be detected in all matter in concentrations ranging from less than 0.1 $\mu g/m^3$ in the air to 4 g/kg in soils. This metal is usually present as Cr(III), but hexavalent chromium in the environment is almost totally derived from human activities (WHO, 1990) and used mainly in the form of soluble salts, excessive exposure in the

workplace has shown that Cr(VI) can act as an acute irritant, a carcinogen and an allergen for humans (Dayan and Paine, 2001).

The genotoxic effects of chromium compounds have been extensively studied using different test systems and measuring various targets and/or genetic endpoints (Amrani et al., 1999). Hexavalent chromium compounds of varying water solubilities were consistently active in numerous studies covering a wide range of tests for genetic and related effects, being classified as a carcinogen by International Agency for Research of cancer (IARC). In particular, potassium, sodium and ammonium dichromates and chromates induced DNA damage, gene mutations in a number of targets, including animal cells *in vivo* and animal and human cells *in vitro* (WHO, 1990).

Another ubiquitous element is aluminum; it can be found in every food product, and is among the most common elements on our planet (Williams, 1999). The sources of aluminum are especially corn, yellow cheese, salt, herbs, spices, tea and tap water. This metal ion may cause diseases in human, by targeting some metabolic process, such as the calcium, phosphorus and iron turnover (Ochmanski and Barabsz, 2000). It has been investigated the accumulation of aluminum in the brain tissue of patients with Alzheimer`s disease and the Parkinsonian dementia (Alshuaib and Mathew, 2005; Yousef, 2004). Complexes of this trivalent ion with oxalate can cross the plasma membrane of some cells and is deposited in vacuoles vesicles (Williams, 1999).

The last metal ion to consider is lead; it is used in many industries, including lead melting and processing, the manufacturing of batteries, pigments, solder, plastics, cable sheeting, ammunition and ceramics. Most of cases of lead poisoning in adults result from occupational exposure, although lead exposure in general population is primarily through diet. This has many effects in the body. One the best studied is the impact on hemoglobin synthesis, inhibiting several of the enzymes in the heme formation pathway (Gochfeld, 1997).

Within this frame, and in order to analyze the toxic activity of chromate ion, aluminum and lead, this study used low concentrations of these metals, to analyze the accumulative effect in some biology aspects of *D. melanogaster*, including carboxylesterase activity, behavior, weight and egg viability.

Materials and Methods

Chemicals and Culture Media

Aluminum chloride, potassium dichromate and lead nitrate were used in a final concentration of 50μM, dissolved in distilled water and added to the banana-agar culture medium (banana, agar, water, sugar and nipagin as fungicide). In order to avoid the change of oxidative state of the chromium, the mixture was prepared under controlled temperature, approximately 45ºC. About 30 mL of this medium were transferred to 250 mL glass bottles and used to feed the fruit flies.

Stocks

Specimens of *Drosophila melanogaster* were collected using traps (Medeiros and Klaczko, 1999) in May 2005 at São José do Rio Preto, State São Paulo, Brazil. One isofemale stock was created, formed by one female. The insects and the experimental cultures were maintained in a temperature-controlled chamber at 25 ± 1ºC.

Generations

For each experiment the flies were maintained in a contaminated medium culture with the metal for ten generations. In the formation of the first generation, 12 recently emerged couples were removed from the isofemale stock. For each metal and for the control (without metals) we did three replicates. After five days of the first adult emergence, 12 new couples were removed to start a new generation, and this whole procedure was repeated for 10 generations.

Experiments evaluating viability and development

Female and male flies from the isofemale stock were collected immediately after emergence and subsequently kept separated into tubes with culture medium for five days. In the sixth day one male and one female were put in contact in bottles containing standard or treated medium culture for one day. Afterwards the male was removed and the female laid eggs for 24 hours in a spoon containing agar-sugar medium. The eggs were counted and the spoon was transferred to a bottle containing control or treated culture medium. Subsequently the pupal cases and the number of emerged adults were scored. This procedure was done in triplicate in F_3 and F_{10} generations.

Effects on weight

The progenies of F_1 and F_{10}, from the isofemale strain, were weighted using an analytical scale. We utilized adults recently emerged (3 hours). They were separated by sex for six days in 1.5 mL tubes with banana-agar medium and weighted daily in an analytical balance AND HR-200.

Effects of different concentrations

We analyzed the effect of three concentrations: 25, 50 and 100 µM, on productivity. One female and one male from F_8 were allowed to couple for one day in a tube containing culture medium (control or containing a metal), after, the male was removed and the female was allowed to lay egg for three days in the same tube. This female was transferred to a new tube containing the same culture medium, and this was repeated for other seven times. The offspring was scored in each tube. Three replicates of each concentration were done, and for the control group, nine.

Mating tests

This was an analysis of the flies' behavior, used to measure the pre copulation and copulation times. Adults from F_1 and F_{10} of the isofemale stock were separated by sex in groups of five, for six days in tubes containing culture medium (control or containing a metal). Then, the females and males from each group were transferred, without anesthesia, to a tube without the culture medium. In each vial the males and females had the same exposure history. Each vial was observed for 60 minutes, to determine the pre and copulation times using a digital chronometer, as performed by Itoyama (1992).

Assay of carboxyl esterase activity and total protein quantification

Carboxilesterase activity was measured according to the method of Ellman et al., (1961), following the release of thiol-derivatives at 412 nm on a Varian Cary 100 spectrophotometer, and using phenylthioacetate as substrate (Bonacci et al., 2004; Vioque-Fernandez et al., *in press*). Pools of five animals were homogenized in 200 µL of Tris-HCl 100 mM buffer, pH 8,0, and centrifuged at 10,000 xg for 15 min. using a Jouan CR3i centrifuge, at 4 ^{o}C. Assays were carried out adding aliquots of the supernatant fraction to a cuvette containing the homogenization buffer with 1 mM DTNB final concentration. Blanks without substrates or samples were previously

incubated at 25 ºC for 2 min to assess endogenous cross-reaction with DTNB. Assays started by addition of the substrate phenylthioacetate (4,5 mM final concentration). Specific activities are expressed as U mg protein^{-1}; one unit is the amount of enzyme hydrolyzing one µmole substrate min^{-1}. Protein concentration was determined by the method of Bradford (1976). Absorbance readings were carried out in a Cary 100 Varian spectrophotometer.

Statistical analyses

In order to determine whether the treated and control groups has significant differences, we applied the Student´s *t* test (Zar, 1999). Differences with $p<0.05$ were considered as significant. Calculations were carried out using BioEstat 4.0 program (Ayres et al., 2005).

Results

The first experiments analyzed the metal effects on the eggs' viability, investigating the effects at each stage of the developmental cycle. In order to observe the accumulative effect, the used females and males were from F_3 and F_{10}.

As shown in Table 1, in F_3 the number of laid eggs showed a decrease for all the groups exposed to metals. The highest effect was produced by lead, which decreased the egg to adult viability in 21%. The number of pupae and adults decreased about 4 times between the control, on one side, and the groups exposed to the metals ions on the other. A remarkable point is that the toxic effect for F_3 targeted especially the eggs, since practically all the pupae emerged as flies, with the only exception of the group exposed to lead, which presented toxic effects also at the pupa-adult process.

After seven generations of constant contact with metals ions, in F_{10}, the values changed, compared to F_3. Considering the absolute numbers of laid eggs, the largest decrease affected the control group. Reductions occurred also for the groups exposed to lead, while an increase in pupal-adult was evident for the replicates exposed to chromium.

Comparing only the groups analyzed in F_{10}, the viability from eggs to pupae decreased for lead and increase for aluminum and chromium in F_{10}, including the control, which had an average smaller than two of the exposed groups (Al and Cr). The eggs exposed to lead were significantly affected, decreasing their viability to 53%.

Differently from F_3, the viability from pupae to adults was in F_{10}, lower for aluminum and lead treatments, decreasing in average around 5% for the control group.

Table 1: Egg viability for three different treatments: aluminum, chromium and lead. The eggs, pupae and adults were scored in F3 and F10 from the isofemale line in each treatment and in the control.

Gen	Treatment	Control	Aluminum	Chromium	Lead
	Eggs	46.7±12.6	16.3± 10.3	9 ± 7.1	12 ± 0
	Pupae	46 ± 9.5	13.3 ± 9.7	9 ± 7.1	10 ± 1.4
F3	Adults	46 ± 9	13.3 ± 9.7	9 ± 7.1	9.5 ± 2.1
	Viability Egg-adult (%)	95.5 ± 6	84.6 ± 23.2	90 ± 14.1	79.2 ± 17.7
	Viability Egg-pupal (%)	96.1± 5.1	84.6 ± 23.4	90 ± 14.1	83.3 ± 11.8
	Viability Pupal-adult (%)	99.4 ± 1.0	100 ± 0	100 ± 0	94.4 ± 7.8
	Eggs	21.5 ± 3.5	19.3 ± 7.6	19 ± 2.8	15 ± 0
	Pupae	16.5 ± 0.7	15.7 ± 5.5	16 ± 2.8	8 ± 0
F10	Adults	15.5 ± 2.1	15 ± 6.1	16 ± 2.8	7 ± 0
	Viability Egg-adult (%)	72.2 ± 2.	77.4 ± 2.1	84 ± 2.4	46.67 ± 0
	Viability Egg-pupal (%)	77.5 ± 9.5	81.8 ± 3.6	84 ± 2.4	53.33 ± 0
	Viability Pupal-adult (%)	93.7 ± 8.8	94.7 ± 4.6	100 ± 0	87.5 ± 0

Since the weight of the insects could reflect toxic effects, we determined the mass of adults that emerged in F_1 and F_{10}. As shown by figure 1, in the first generation only aluminum had an increase in the mean weight, whereas for chromium and lead the values were lower than for the control, although these differences were not significant. In the last generation, F_{10}, the mean weight in control, as well as in lead exposed group increased, when compared to the first generation, but these differences were not significant. The groups exposed to aluminum and chromium had a decrease of about 50% in the mean weight of the control group.

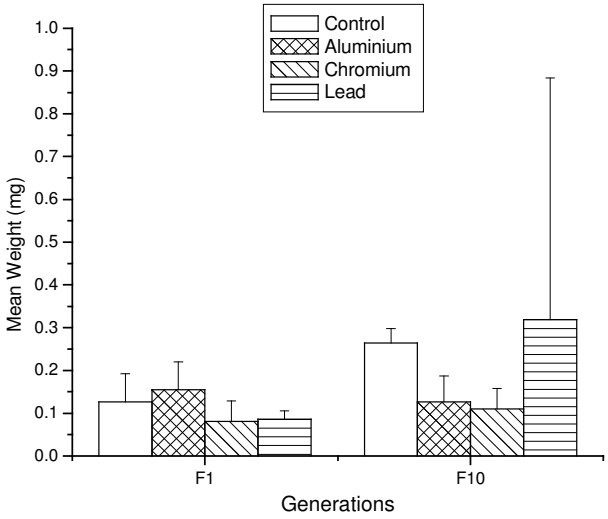

Figure 1: Average weight ± S.D. of adult females and males in two generations (F_1 and F_{10}) of the isofemale line of *D. melanogaster* exposed to aluminum, chromium and lead. Each treatment was carried out in triplicates.

We tried to analyze the neurotoxicity from different points of view, the time of pre copulation and copulation (expressed as mean proportion) was evaluated for couples submitted to the same element. As observed in figure 2, the average of pre-copulation time was different for each metal. The treatment with aluminum had a mean time similar to the control in F_1 and F_{10}. In F_5 it presented a 63% increase compared to the control group. For chromium, the mean pre copulation time was higher to control in F_1 and F_{10}. On the other hand, for lead, the pre copulation time had a non significant increase in all the observed generations.

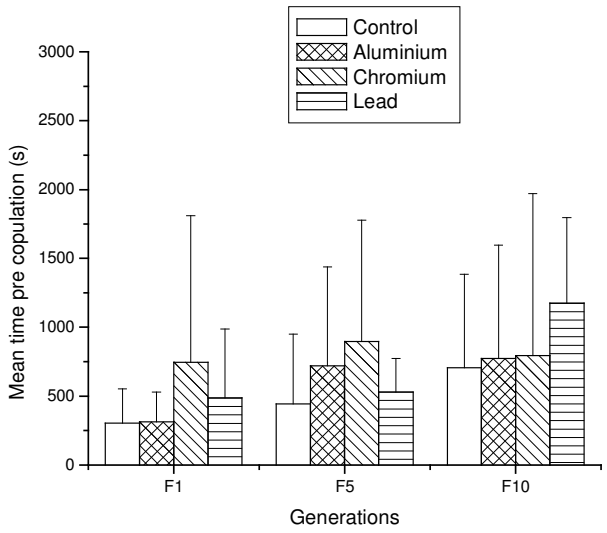

Figure 2: Average of pre-copulation time in three generations of the isofemale line of *D. melanogaster* (F_1, F_5 and F_{10}) exposed to aluminum, chromium and lead.

The effect of each metal ion in the copulation time was analyzed (figure 3), and the results were different from the pre-copulation time. In F_1 and F_5 all the exposed groups had a similar mean copulation time, about 5% lower than the control one. Analysis in F_{10} showed an increase for the mean pre copulation time for aluminum and chromium. For chromium this difference was significant, even though this difference was lower than in the pre copulation analysis. For the group exposed to lead, the mean time copulation decreased more.

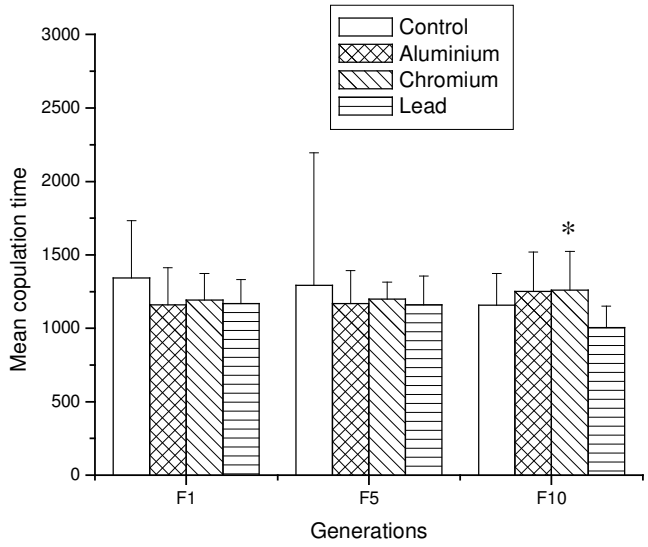

Figure 3: Mean copulation time in three generations (F_1, F_5 and F_{10}) exposed to aluminum, chromium and lead. Asterisk indicates $p<0.05$.

Biochemical analysis focused in the carboxylesterase activity. It was measured with groups of five adults from each metal compound. As shows figure 4, the group exposed to aluminum had an increased enzyme activity compared to the control group. For the other groups the activity was similar to that found in the control. It is interesting that for chromium the variation was minimum, indicating higher homogeneity of the group and in the Student's *t* test this difference was significant.

The last experiments involved an analysis of different metal concentrations (25, 50 and 100 μM). A couple from F_8 exposed to a metal concentration of 50 μM, was transferred to vials with a different or equal concentration. The results are shown in figure 5. For the insects exposed to aluminum, the lower concentration (25 μM) reduced the average emergence, and for 50 μM the emergence was increased comparing with control. When the concentration doubled (100 μM), the emergence reached the same value of the control group. The experiment with chromium showed that emergence was

not affected with increasing concentration of the product. In the group exposed to lead, the average emergence increased only for the highest concentration.

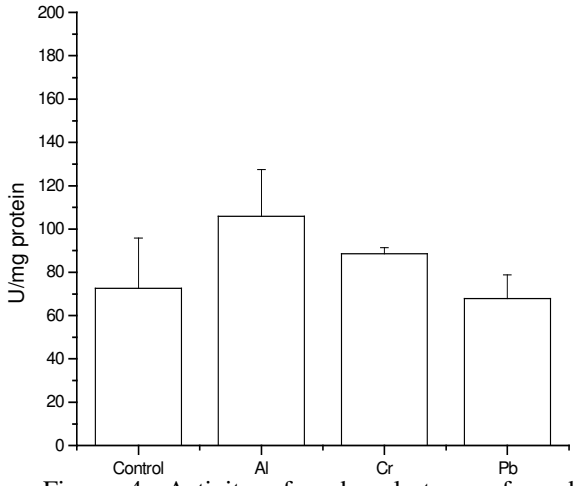

Figure 4: Activity of carboxylesterase for adults from F_5 exposed to aluminum, chromium and lead.

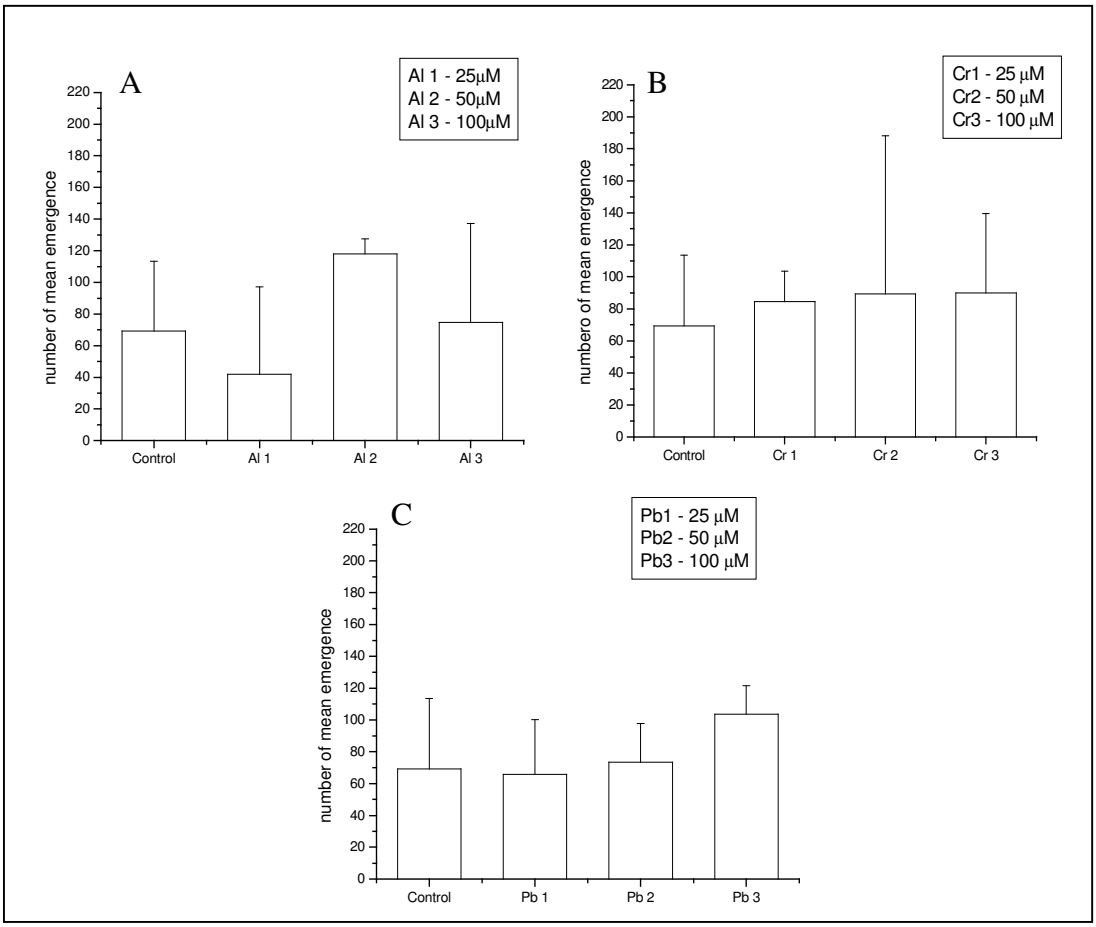

Figure 5: Mean emergence of adults from F_8 of the isofemale line submitted to different metal concentrations, 1- 25 μM, 2- 50 μM and 3 – 100 μM. A- Graph for aluminum; B- Graph for chromium and C- Graph for lead. The values are the average of three replicates for the exposed groups and nine for the control.

Discussion

Physiological imbalance of metals due to genetic disorders or environmental pollution is a worldwide problem associated with adverse health effects. There is a number of genes and regulatory mechanisms involved in heavy metal homeostasis. Disequilibrium of cooper and zinc, for example, can lead to disease and poses a significant health risk to humans and animals (Yepiskoposyan et al., 2006).

Analysis performed for lead, showed that, in the experiments of egg viability (Table 1), this product affected the stages of *Drosophila* cycle in the two analyzed generations. It was already reported that lead acetate (1.2 mM) in *D. melanogaster* affects the time course of pre-adult development (Akins et al., 1992). In agreement with Uysal and Bahceci, (1996) lead has been reported to reduce fecundity (0.05 to 5.0% of lead acetate).

In the behavioral studies (Figures 2 and 3) the females and males exposed to lead had more differences in the pre and copulation times compared to control group. Increasing the pre copulation time according generation passage, and decreasing copulation in F_{10}, although the difference was not significant in statistical analysis. The literature reveals that *Drosophila* accumulates lead and this product is neurotoxicant. Exposure to this chemical product can have significant effects on the number of females that mate within 20 minutes and also on their fecundity (Hirsch et al., 2003). Lead is neurotoxic; it produces changes at the synaptic connections between neurons in both mammals and flies (Hirsch et al, 2003). In humans, lead exposure is known to affect children development, provoking changes in attention and cognitive function (Walkowiak et al., 1998; Lanphear et al., 2000).

Weight investigation in flies lead presented a weight reduction in the first generation and a mass increase in the last generation. However, the changes provoked by lead in terms of developmental delays, are not accompanied by changes in the adult body weight, indicating that modifications in long term nutritional status of larvae are not the underlying cause (Akins et al., 1992).

In terms of carboxylesterase, a serine-dependent esterase that hydrolyzes a wide range of xenobiotic substrates (Bonacci et al., 2004), its activity was unaffected. It is known that lead inhibits several enzymes in the heme formation pathway (Gochfeld, 1997) but nothing was found about esterase system activity related in lead toxicity.

Concerning the experiment done with different concentrations, lead affected the emergence number of the group exposed to 100 μM (Figure 5). According to Akins et

al. (1992), the toxicity of lead in *Drosophila* is known to be dose-dependent; in this case, the response to the presence of the metal was an increase of the offspring.

The other metal investigated was chromium. Studies revealed that $CrCl_2$ and CrO_3 decrease the fidelity of DNA synthesis *in vitro* (Sirover and Loeb, 1976). This finding is interesting because the capability of a compound to produce infidelity during polymerization is correlated to its mutagenic and carcinogenic properties (Loeb et al., 1974; Mizutani and Temin, 1976). Specifically in *Drosophila*, $CrCl_3$ (0.25μM), in the nutritive medium had intensive effects on puff formation in *D. melanogaster* (Nikiforov et al., 1970). Studies with *D. melanogaster* showed that genetic alterations induced by Cr^{+6} and Cr^{+4} appear to arise from induced mitotic recombination; it is likely that the initiation event in chromium carcinogenesis involves the double strand excision of the DNA helix leading to a loss of heterozygosity in various targets sites in the nuclear genome (Katz et al., 2001).

In assays of somatic mutation and recombination tests (SMART) with *D. melanogaster*, Cr (VI) showed to be genotoxic, inducting high levels of mitotic recombination (Graf et al., 1992). Recently, it was demonstrated that chromium picolinate generate heritable genetic changes and delays in progeny development in *Drosophila melanogaster* (Stallings et al., 2006). In our study with egg viability chromium had more effect in the last generation, so it is an indicative of accumulative process.

The effect of chromium on the pre copulation and copulation times (Figures 2 and 3), demonstrated that pre copulation time increased in the first exposition, and the copulation time only increased significantly in the last generation compared to the control group. The study of viability (Table 1) showed that chromium in the last generation analyzed, improved slightly the emergence as adults. In 2003 Hepburn et al. discussed that in all instances, larva exposed to chromium picolinate exhibited a diminished ability to pupate, reduced pupae viability, and dose-dependent delays in reaching the developmental milestones of pupal formation and eclosion.

However, the effects on larval viability, although always apparent, were somewhat variable from experiment to experiment. It is important to note that Levinson and Bergmann, 1959, found for *Musca vicina* that chromium as picolinic acid affected larvae survival, weight, and success of pupation and also ultimately the success of larvae developing into adults in a deleterious way.

Concerning the effect on body mass (Figure 1), the groups exposed to chromium had a higher average weight (not significant) compared to the control group in F_1 and F_5. In humans, it was postulated that chromium dietary supplementation has effects on body composition, including reducing fat mass and increasing lean body mass (Hepburn et al., 2003).

In relation to carboxylesterase, the activity was similar to the control, but other enzymes can be involved in this process. Glutathione and cysteine seem to be the most important cofactors for the intracellular reduction of hexavalent chromium, but ascorbic acid, microsomes in the presence of NAD/NADH, microssomal cytochrome P450, mitochondria and proteins such as hemoglobin and glutathione reductase may also be active in the reduction process (Dayan and Paine, 2001).

The last element analyzed was aluminum; it is a trivalent ion difficult for biological systems to handle, which tends to participate in slow reactions, especially of phosphates, in all organisms (Williams, 1999). It is known for its neurodegenerative effects in human, related to diseases like Alzheimer (Mailloux and Appanna, 2006). This element affected significantly the viability eggs-adults of F_3.

Concerning the behavioral test, only pre copulation mean time was altered (Figure 2). In F_1 and F_{10} it was similar to the control, but in F_5 an increase was observed. Aluminum inhibits delayed-rectifier potassium current in *Drosophila* neuron and reduced the voltage-dependent influx of calcium into synaptossomes and smooth muscle cells in rabbits and cats. In *Drosophila* this reduction also increased calcium influx via voltage-gated calcium channels, and this into the presynaptic nerve terminal can directly enhance neurotransmitter release (Alshuaib and Mathew, 2005). It could explain the increasing pre copulation time.

The carboxylesterase assays of exposed insects showed that the activity was higher than for the control group, but not significant. Classically, pollutants are known to cause esterase inhibition, but a stimulatory effect has also been reported for substances such as lindane (Muñoz-Blanco et al., 1985), toluene, vinyl chloride, the organophosphate ethylparathion (Sanz and Repetto, 1995), and also metals, like lead (Bainy et al., 2006) and aluminum (Zatta et al., 2003). Romani et al. (2003) observed increased acetylcholinesterase activity in the fish *Sparus auratus* exposed for 20 days to sublethal copper concentrations, proposing that copper could enhance the formation of the enzyme-substrate complex, increasing the activity of AchE (acetylcholinesterase). Another possibility for esterase activation by metals would also be related to a *de novo*

synthesis of the enzyme as a response to an initial inhibition. In fact, a differential activation of multiple molecular forms of the esterase system has been reported previously for the shrimp *C. thyrrena* after Hg exposure (Thaker and Haritos, 1989). In general, it has been related that metals cause esterase activation after acute exposures, and esterase inhibition after chronic exposures. Gallegos et al. (2001) observed an increase on the acetylcholinesterase activity in brain of rats exposed to 10 mg/kg of lead, after 30 min, but a strong decrease in this enzyme after 24 and 72 exposure hours. Thus, the tendency of carboxilesterase increases observed in our work for those animals exposed to aluminum are in agreement with previous reports on esterase behavior in response to different metals. In the 25 μM (minor concentration) the emergence was the lowest too, but the higher concentration had the same emergence of the 50 μM group.

Toxicity of aluminum comes from the substitution of magnesium ions in the cellular growth. The neurotoxic action of this metal probably comes from substitution of Mg ions in ATP, what finally influences the function of every ATP using enzymes. Although most organisms succumb to the toxic influence of aluminum, some living systems are known to elaborate intricate strategies to fend off the dangers associated with an aluminum-rich environment (Middaugh et al., 2006).

This study showed that the three metals showed influences in the biology of *Drosophila*, despite of their low concentration. However, it is important to remember that the genetic mechanism of heavy metal resistance in *Drosophila* is a highly complex and dynamic system, and heavy metals may directly (via germ cell mutations) or indirectly (via somatic mutations or physiological and ecological effects) change populations (Jordaens et al., 2006). To gain a deeper understanding of the real effect in of heavy metal in living organisms it is important to know their toxicokinetics in different bioindicators and populations.

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DISCUSSÃO GERAL

Capítulo Seis

DISCUSSÃO GERAL

A probabilidade de exposição dos seres humanos e do meio ambiente a substâncias químicas é cada vez maior pelo aumento no seu uso em indústrias e no seu volume de produção. Entretanto, em muitos casos não há informação sobre os riscos tóxicos dessa exposição, em especial no que se refere aos efeitos da exposição crônica (CALAFAT et al., 2005). Além disso, os efeitos de xenobióticos são individuais, baseados nos fatores fisiopatológicos e interações ambientais, com forte influência das características genéticas. Em muitos casos a toxicidade depende da concentração no sítio de ação (CASCORBI, 2006), não havendo uma relação direta entre a concentração da exposição e os efeitos tóxicos a serem observados.

Isso foi verificado nos experimentos de diferentes concentrações de acrilamida, coomassie e metais testados, nos quais verificamos que, em termos de número de emergentes, nem sempre a maior concentração foi a de maior efeito. No caso do cloreto de alumínio, a menor concentração (25 μM) foi a que apresentou menor emergência. Entretanto, para o nitrato de chumbo, com o aumento da concentração houve um aumento na média de indivíduos que emergiram. Já para o dicromato de potássio, não houve influência alguma da concentração do produto químico no meio de cultura.

Com relação aos experimentos de comportamento para avaliação da neurotoxicidade, é importante lembrar que segundo Ashburner e Canton (1983) quanto à corte e a cópula, estas ocorrem em períodos quando as moscas estão se alimentando. Os machos cortejam as fêmeas que, se receptivas, produzem um estímulo apropriado, o qual desencadeia as demais seqüências da corte. As ações de corte das fêmeas são mais limitadas em número e diversidade do que as dos machos, as quais são produzidas pelos movimentos das asas, patas, genitália e abdômen. As fêmeas podem reagir de duas formas ao macho: rejeitando-o ou aceitando-o, e é comum a fêmea andar ou se

alimentar durante a cópula. A duração desse comportamento varia de 30 minutos a 1 hora (ASHBURNER e CANTON, 1983). Segundo alguns pesquisadores os machos de *D. melanogaster* atingem um máximo de atividade de corte durante a segunda metade da noite e as fêmeas podem produzir de 10 a 20 ovos, mas em condições apropriadas de laboratórios, chegam a 100 ovos por dia, o que corresponde a seu próprio peso (DAVID, 1970; COHET e DAVID, 1978)

Assim, de acordo com os experimentos de tempo de pré-cópula e cópula, os únicos eventos em que foram observadas diferenças significantes ocorreram com o chumbo e cromo.ambos na geração F_1. O tempo de cópula apresentou uma diminuição quando comparado ao controle. Para os demais produtos ou metais testados, ocorreram diferenças nos tempos de pré-cópula e cópula que não foram significantes.

Para alguns metais utilizados como o chumbo e cromo as variações observadas no experimento de viabilidade são semelhantes aos relatados na literatura (HEPBURN et al., 2003 e HIRSCH et al., 2003), mas para o estágio larval. A análise das porcentagens de ovos que chegaram à fase de pupa e de pupas que emergiram em adultos, revelou que a acrilamida foi o produto mais tóxico, afetando o ciclo de vida, nas duas gerações analisadas (F_3 e F_{10}). Dessa forma, em F_3 somente 45,1% dos ovos eclodiram em adultos e em F_{10}, esse índice foi de 40,7%.

É importante ressaltar, para os metais testados, que vários íons metálicos são vitais para muitos processos biológicos, tais como catálise enzimática, transcrição, respiração e crescimento. No entanto, o acúmulo desses, mesmo que essenciais, é prejudicial à homeostase dos metais podendo levar a doenças ou aumento do risco de saúde tanto em animais, quanto no homem (YEPISKOPOSYAN et al., 2006). Alguns dos metais utilizados neste projeto possuem função biológica para plantas e animais, como por exemplo, o cromo que parece estar envolvido no metabolismo da glicose. Já o

chumbo e o alumínio não têm função conhecida (Guilherme e Marchi, 2007; Williams, 1997).

Além disso, a suscetibilidade aos metais pode ser analisada em termos individuais ou populacionais e pode ser quantificada como a probabilidade ou sinal de resposta de um evento ou exposição particular (GOCHFELD, 1997). No caso deste trabalho, a quantificação dos sinais advindos da exposição contínua aos produtos químicos foi realizada pelo acompanhamento das gerações contabilizando os indivíduos adultos, além dos experimentos ao nível de enzimas e de comportamento.

Com relação ao aspecto populacional, a suscetibilidade ao agente químico ou biológico pode ser representada pela sua distribuição, entretanto esse padrão é raramente conhecido. A suscetibilidade a qualquer agente é resultado da interação entre genética (polimorfismo genéticos, por exemplo) e ambiente (nutrição, exposição passada, aptidão, entre outras) (GOCHFELD, 1997). A resistência a metais pesados em *Drosophila*, por exemplo, é um processo complexo e dinâmico que afeta a freqüência de inversões, e aumenta as interações epistáticas com todo o genoma como no caso do estudo do efeito do chumbo em *Drosophila subobscura* (KALAJDZIG et al., 2006).

Essa interação entre genética e ambiente no processo de toxicidade pode ser verificada no estudo comparativo das linhagens, onde mesmo o grupo controle parece ter sido afetado pelos endocruzamentos durante as dez gerações. No grupo dos tratados, para cada tipo de produto químico utilizado houve uma resposta diferente com o passar das gerações, ou seja, aumento e diminuições na média da emergência dos adultos.

Ugolini et al. (2004), no estudo conduzido com o crustáceo *Talitrus saltator* reportaram que havia uma correlação positiva entre o grau de diversidade genética e o grau de contaminação por metais pesados. Recentemente Jordaens et al. (2006) em estudo conduzido com os crustáceos *Cepaea nemoralis* e *Succinea putris* revelou que

mudanças genéticas nestas populações deveriam ser resultado da ação do "gargalo da garrafa", da seleção mediada pela poluição ou a combinação desses fatores, mas que não haveria indicativos da presença de alelos de resistência à poluição ou de modificação pós tradução ou pós transcrição. Dessa forma, nos estudos relacionados à poluição devem ser considerados os aspectos relacionados com a genética e a resistência, os quais estão sendo investigados.

Na tentativa de verificar o significado ecológico de mudanças induzidas pelos produtos químicos e metais na cópula e na fecundidade foram formuladas algumas questões relacionadas principalmente à contaminação com chumbo, mas que seriam válidas para todos os tipos de contaminação que envolvem mudanças no comportamento.

Segundo Hirsch et al. (2003), o aumento na freqüência de cópula e fecundidade nas populações de *D. melanogaster* tratadas com chumbo, pode ser interpretado em termos de mudança de alocação de recursos pela fêmea. Há três possibilidades de interpretação. A primeira, quando as condições ambientais são favoráveis, as fêmeas podem ser mais seletivas na escolha do parceiro e podem investir intensamente na prole individual (mais ovos) maximizando sua qualidade. Em contraste, em ambientes adversos a fêmea pode aumentar a freqüência com a qual copula e/ou o número de ovos (menores), desse modo o aumento na diversidade genética de sua prole pode fazer com que algum deles seja bem adaptado a estas condições adversas.

Uma segunda possibilidade, ainda de acordo com Hirsch et al. (2003) afirma que as fêmeas podem no ambiente adverso não se reproduzir até que as condições melhorem, ou seja, até que sejam transferidas para um meio de cultura controle (sem o produto químico). Em ambos os casos, a fêmea estaria usando as informações sobre o ambiente, para mudar suas estratégias reprodutivas, influenciando a composição da

próxima geração. A terceira hipótese baseia-se na oviposição indiscriminada em termos de quantidade, no meio de cultura. Contudo, não acreditamos que o fenômeno da elevação da emergência seja explicável pelas hipóteses acima. Na continuidade deste trabalho serão feitos experimentos procurando uma explicação plausível.

A análise das duas linhagens revelou que na décima geração da linhagem massal houve um aumento do número médio de emergentes com relação à análise da geração anterior. Fato que foi verificado como diminuição dos emergentes na isolinhagem. O aumento do número de emergentes pode ser atribuído a maior variabilidade da linhagem massal, uma vez que foi formada a partir de vários casais. Enquanto que a diminuição observada na isolinhagem, poderia no mesmo sentido, ser explicada pela menor variabilidade genética da linhagem, formada a partir de uma única fêmea.

A análise dos perfis de emergência dos produtos químicos e metais testados durante as dez gerações revelou que na primeira geração sempre a média do número de indivíduos emergentes foi maior para o grupo dos tratados que para o controle, ou seja, na presença dos contaminantes as fêmeas estariam alocando mais recurso para a produção da prole. Entretanto os testes de massa corpórea mostraram que exceto para cromo e chumbo, após sucessivas exposições ao produto químico testado (F_{10}) os indivíduos tiveram perda de massa em comparação com o controle.

Por sua vez, o acompanhamento do ciclo biológico indica que para alguns casos como o do coomassie, a presença do xenobiótico aumentou a viabilidade dos ovos, ou seja, a porcentagem de ovos que eclodiram em adultos foi maior para os tratados que para o controle. Já com relação aos aspectos analisados, contagem de adultos, freqüência de alterações, tempo de pré-cópula e cópula, atividades enzimáticas e massa corpórea, a acrilamida foi o produto que ocasionou efeito em todos os itens, por outro lado, o coomassie foi o produto que causou o menor impacto.

Embora muitos dos produtos químicos testados já tenham sua carcinogenicidade ou genotoxicidade comprovadas ou avaliadas pela IARC (Agência Internacional de Pesquisa sobre o Câncer), muitas das reações decorrentes da presença destes no organismo, podem apresentar tanto efeitos sinérgicos como antagonistas, apresentando resultados distintos dos esperados convenientes pela toxicologia (SIDDIQUE et al., 2005). Como pode ser observado nos resultados dos anexos 1, 2 e 3, onde o aumento do número de emergentes no grupo dos tratados foi relatado na maior parte das gerações analisadas. De acordo com o anexo 4, depois de dez gerações de exposição contínua a cada produto químico, houve um aumento no número de emergentes totais para os tratados, principalmente para a exposição ao dicromato de potássio.

Os resultados obtidos foram com moscas de no máximo um dia de vida, e de acordo com Jacobson et al. (1983), há a possibilidade de que estas moscas tenham consumido menos contaminantes que aquelas com melhor tempo de vida, em média, diminuindo o efeito do produto tóxico. Para se chegar a uma conclusão a respeito da toxicidade, é necessário considerar, além das ações no organismo, todo o processo de toxicodinâmica, ou seja, é preciso conhecer o mecanismo de ação da substância química para identificar quais são os efeitos adversos decorrentes daquela ação (AMORIM, 2003).

CONCLUSÃO

Capítulo Sete

CONCLUSÕES

O presente trabalho permitiu analisar os efeitos de baixas concentrações de diversas substâncias utilizadas de forma rotineira em laboratórios de pesquisa da área química e biológica. A avaliação básica procurou investigar os efeitos sobre os níveis de produtividade diária ao longo de 10 gerações de duas diferentes linhagens: uma massal e outra isofêmea. De forma simultânea ao acompanhamento da emergência de cada geração, realizou-se uma análise morfológica das principais alterações morfológicas observadas nos adultos. Adicionalmente, foram feitos experimentos de comportamento (tempos de pré-cópula e cópula), viabilidade ovo-larva-pupa-adulto, atividade de carboxilesterase e o efeito de diversas concentrações da substância química sobre a produtividade. A seguir faremos um resumo das observações:

- Para a acrilamida observamos uma resposta dose-dependente entre a concentração deste produto no meio de cultura e a média de emergência de *D. melanogaster*. Houve também redução da viabilidade dos ovos em 50%. Nos testes de comportamento, houve aumento no tempo médio de pré-cópula, sem alterações evidentes no tempo de cópula. Quanto à produtividade, medida com base no número de emergentes, em 10 gerações não detectamos um efeito pronunciado da acrilamida embora em algumas situações específicas tenhamos verificado um aumento no número de emergentes submetidos à acrilamida e um deslocamento do(s) pico(s) de emergência

- Para o Alumínio, ao longo de dez gerações de exposição contínua, ocorreu redução no número de ovos, mas as viabilidades de emergências e eclosão mantiveram-se semelhantes às do controle. Não houve diminuição no número de

indivíduos após dez gerações submetidas a esse metal. Quanto ao padrão de emergência parece haver um deslocamento de máximo de emergência. O alumínio também foi o metal que causou a maior freqüência de alterações morfológicas. Em algumas gerações, por exemplo, F_5, foi observado aumento do tempo de pré-cópula, além de aumento da atividade da carboxilesterase.

- Os principais efeitos observados na exposição ao cromo hexavalente após dez gerações de exposição foram diminuição do peso e aumento significativo do tempo de cópula. O número de descendentes nas gerações foi maior no grupo tratado com cromo comparado ao controle. Não houve uma resposta dose dependente entre a concentração do produto no meio de cultura e a média de emergência.

- O chumbo, juntamente com a acrilamida, foi o produto que mais afetou a viabilidade dos ovos; cerca de 50% deles não chegaram ao estágio de pupas. Metade das gerações expostas ao nitrato de chumbo apresentou aumento na emergência comparado ao controle, sendo a diferença em F_1 significante. As alterações morfológicas foram freqüentes, destacando-se na quarta e na sexta gerações, nas quais, segundo a literatura há aumento de inversões cromossômicas em *D. subobscura*. Na última geração houve também aumento do tempo de pré-cópula. Além disso, no experimento com diferentes concentrações, a mais elevada apresentou a maior emergência média, quando comparado com o controle.

- O Coomassie Brilliant Blue G-250 foi o produto que menos afetou a biologia de *D. melanogaster*. Em nossos experimentos em metade das gerações analisadas houve aumento na proporção de emergentes em comparação com o controle, sendo que na sétima geração esse aumento foi significante. Os testes de comportamento não apresentaram diferenças significantes, apenas houve aumento do tempo de pré-cópula que se acentuou com o passar das gerações. É interessante ressaltar que a viabilidade dos ovos foi aumentada para com a exposição ao corante.

Os resultados mostraram que todas as substâncias testadas exerceram algum tipo de resposta mensurável em *Drosophila melanogaster*, embora em diversos graus de magnitude. A variedade de substâncias testadas no projeto permitiu caracterizar a abordagem que utilizou como bioindicador a *D. melanogaster* como sendo válida e capaz de refletir de forma eficiente a toxicidade de qualquer substância passível de mistura com o meio de cultura.

Os agentes químicos testados, cada um com seu mecanismo de toxicidade intrínseco, provavelmente causaram algumas alterações no DNA, na atividade de enzimas, na expressão de proteínas, no comportamento, e outras não consideradas aqui, que se traduziram nas variáveis mensuradas. Ficou evidente, também, a amplitude de respostas individuais dentro de uma população submetida à mesma condição experimental. Questões levantadas por este trabalho, como por exemplo, os mecanismos de toxicidade e defesa, os efeitos sobre o material genético, a sensibilidade diferente em cada estágio do ciclo de vida, merecem aprofundamento, constituindo objetivos no prosseguimento desta pesquisa.

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ANEXOS

Anexo 1 – Tabela de valores totais de emergência para os metais

Anexo 2 - Tabela de valores totais de emergência para a acrilamida

Anexo 3 - Tabela de valores totais de emergência para o coomassie

Anexo 4 – Gráfico do total de emergência das dez gerações analisadas para cada produto químico e metais utilizados

Anexo 1

Tabela com os totais da emergência para as dez gerações durante os quinze dias, para os metais.

		Dias												
Geração	Produto	1	2	3	4	5	6	7	8	9	11	13	15	Total
	Controle	22	101	44	79	120	59	44	11	7	7	1	0	**495**
1	Aluminio	40	174	45	61	83	94	76	50	35	44	15	1	**718**
	Cromo	17	149	62	40	93	98	77	45	29	27	35	48	**720**
	Chumbo	17	107	89	87	179	162	73	46	34	57	46	61	**958**
	Controle	2	55	69	57	78	77	30	4	1	2	60	0	**435**
2	Aluminio	0	61	53	78	198	161	41	27	24	16	82	0	**741**
	Cromo	0	58	75	36	49	111	37	30	23	5	38	1	**463**
	Chumbo	1	40	34	113	122	35	11	0	1	0	35	0	**392**
	Controle	98	112	71	147	76	129	89	50	51	7	1	0	**831**
3	Aluminio	112	110	55	124	139	78	52	33	33	44	15	1	**796**
	Cromo	44	69	73	36	40	101	103	103	96	25	35	48	**773**
	Chumbo	116	94	66	82	45	56	38	37	20	57	46	61	**718**
	Controle	71	38	53	146	147	128	83	59	23	19	1	0	**768**
4	Aluminio	167	76	91	123	87	101	23	2	0	5	0	0	**675**
	Cromo	9	67	27	147	248	131	57	20	10	4	0	0	**720**
	Chumbo	35	36	43	161	207	180	92	36	17	18	27	2	**854**
	Controle	84	154	119	74	90	79	64	61	58	18	32	0	**833**
5	Aluminio	174	116	68	38	71	20	54	24	22	35	5	0	**627**
	Cromo	183	150	56	71	113	90	119	87	94	114	92	49	**1218**
	Chumbo	108	153	116	80	133	96	58	34	26	46	31	29	**910**
	Controle	3	36	13	31	127	161	102	58	60	38	21	5	**655**
6	Aluminio	74	53	70	49	118	173	82	43	39	61	60	28	**850**
	Cromo	0	34	21	58	115	138	93	32	45	59	46	29	**670**
	Chumbo	8	80	20	57	85	56	78	60	49	49	43	27	**612**
	Controle	1	78	43	23	70	82	43	12	3	4	1	27	**387**
7	Aluminio	32	53	44	75	56	42	79	65	29	23	7	0	**505**
	Cromo	41	103	50	71	89	97	64	34	55	75	150	44	**873**
	Chumbo	23	56	72	204	135	48	29	18	14	18	27	31	**675**
	Controle	93	84	95	166	133	137	73	69	49	49	35	10	**993**
8	Aluminio	109	51	186	214	105	37	43	56	51	86	68	28	**1034**
	Cromo	39	36	135	203	210	182	121	65	87	52	72	49	**1251**
	Chumbo	76	43	81	156	89	136	92	91	48	44	35	15	**906**
	Controle	16	23	49	36	36	91	38	53	23	3	12	4	**384**
9	Aluminio	123	46	71	90	39	74	18	64	27	5	25	5	**587**
	Cromo	9	27	50	94	63	112	61	90	63	59	74	59	**761**
	Chumbo	50	49	33	49	27	85	31	53	10	5	30	5	**427**
	Controle	0	2	7	49	35	31	6	16	9	7	7	0	**169**
10	Aluminio	0	34	50	36	33	70	9	27	8	1	0	0	**268**
	Cromo	0	5	13	24	51	172	35	20	7	1	0	2	**330**
	Chumbo	0	1	9	33	34	69	22	30	38	17	17	15	**285**

Anexo 2

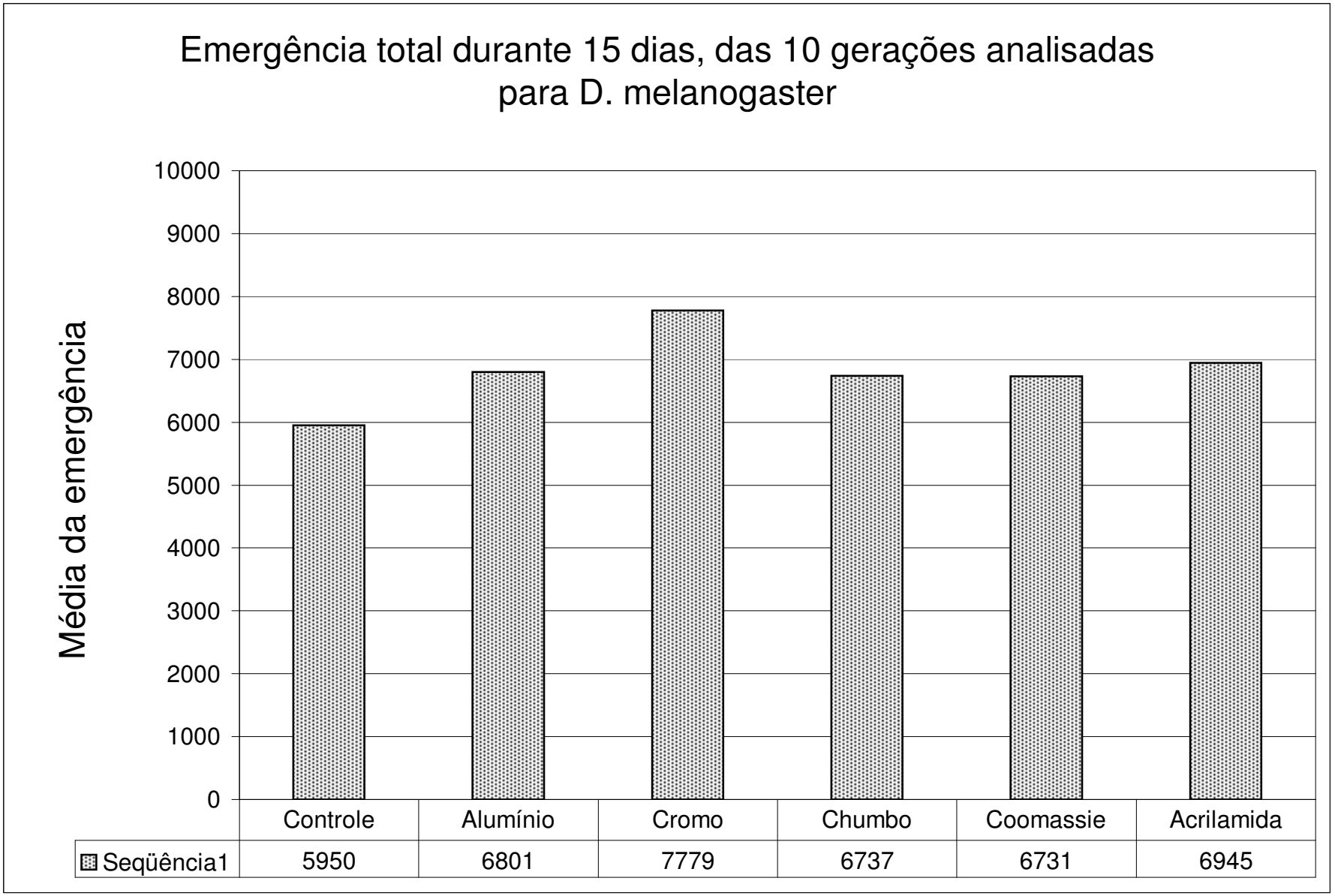
Tabela com os totais da emergência para as dez geações, durante os quinze dias para a acrilamida

		Dias												
Geração	Produto	1	2	3	4	5	6	7	8	9	11	13	15	Total
1	Controle	22	101	44	79	120	59	44	11	7	7	1	0	**495**
	Acrilamida	31	101	73	119	131	69	85	99	49	64	28	46	**895**
2	Controle	2	55	69	57	78	77	30	4	1	2	60	0	**435**
	Acrilamida	5	22	44	85	147	113	65	13	0	5	45	0	**544**
3	Controle	98	112	71	147	76	129	89	50	51	7	1	0	**831**
	Acrilamida	98	88	112	116	29	101	72	45	48	64	27	46	**846**
4	Controle	71	38	53	146	147	128	83	59	23	19	1	0	**768**
	Acrilamida	73	63	90	114	142	99	52	12	11	31	19	15	**721**
5	Controle	84	154	119	74	90	79	64	61	58	18	32	0	**833**
	Acrilamida	55	117	61	52	78	106	96	71	35	56	27	1	**755**
6	Controle	3	36	13	31	127	161	102	58	60	38	21	5	**655**
	Acrilamida	6	47	17	9	38	70	108	96	53	51	99	49	**643**
7	Controle	1	78	43	23	70	82	43	12	3	4	1	27	**387**
	Acrilamida	26	43	92	78	101	77	89	44	81	102	52	31	**816**
8	Controle	93	84	95	166	133	137	73	69	49	49	35	10	**993**
	Acrilamida	32	35	68	138	173	123	91	52	28	51	74	79	**944**
9	Controle	16	23	49	36	36	91	38	53	23	3	12	4	**384**
	Acrilamida	16	47	20	28	23	53	43	45	46	15	23	15	**374**
10	Controle	0	2	7	49	35	31	6	16	9	7	7	0	**169**
	Acrilamida	0	0	22	63	33	43	17	46	30	34	52	67	**407**

Anexo 3

Tabela com os totais da emergência nas dez gerações, durante os quinze dias para o coomassie

		Dia												
Geração	Produto	1	2	3	4	5	6	7	8	9	11	13	15	Total
1	Controle	22	101	44	79	120	59	44	11	7	7	1	0	**495**
	Coomassie	72	130	49	107	92	96	43	35	18	33	19	11	**705**
2	Controle	2	55	69	57	78	77	30	4	1	2	60	0	**435**
	Coomassie	26	97	34	129	117	49	8	1	7	1	57	0	**526**
3	Controle	98	112	71	147	76	129	89	50	51	7	1	0	**831**
	Coomassie	94	192	95	79	73	135	87	49	57	33	19	11	**924**
4	Controle	71	38	53	146	147	128	83	59	23	19	1	0	**768**
	Coomassie	39	79	77	100	169	239	20	12	0	0	3	0	**738**
5	Controle	84	154	119	74	90	79	64	61	58	18	32	0	**833**
	Coomassie	178	162	90	96	109	101	115	79	55	49	50	23	**1107**
6	Controle	3	36	13	31	127	161	102	58	60	38	21	5	**655**
	Coomassie	24	47	17	51	77	138	126	55	37	56	22	8	**658**
7	Controle	1	78	43	23	70	82	43	12	3	4	1	27	**387**
	Coomassie	63	70	45	70	133	96	65	56	50	48	80	26	**802**
8	Controle	93	84	95	166	133	137	73	69	49	49	35	10	**993**
	Coomassie	51	75	97	130	68	75	37	20	39	33	11	11	**647**
9	Controle	16	23	49	36	36	91	38	53	23	3	12	4	**384**
	Coomassie	47	51	46	54	62	62	54	41	15	3	8	3	**446**
10	Controle	0	2	7	49	35	31	6	16	9	7	7	0	**169**
	Coomassie	0	0	7	47	30	44	24	22	3	0	1	0	**178**


Emergência total durante 15 dias, das 10 gerações analisadas para D. melanogaster
Média da emergência
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Controle
Alumínio
Cromo
Chumbo
Coomassie
Acrilamida
Seqüência1
5950
6801
7779
6737
6731
6945

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