

Rita de Cássia Sousa-Polezzi

**Resistência a Inseticidas em Aedes aegypti:  
Modificações nos Padrões de Esterases e  
Ação do Fenobarbital**

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AÇÃO DO FENOBARBITAL**

**Tese apresentada para obtenção  
do Grau de Doutor em Genética.**

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**São José do Rio Preto - SP**

**2002**

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**Orientadora: Profa. Dra. Hermione Elly Melara de Campos Bicudo**

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TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM GENÉTICA.

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Muito Obrigada!!

*Wer glücklich ist, der darf keine Furcht haben. Auch nicht vor dem Tode.*

*Nur wer nicht in der Zeit, sondern in der Gegenwart lebt, ist glücklich. (...)*

*Wenn man unter Ewigkeit nicht unendliche Zeitdauer, sondern Unzeitlichkeit versteht, dann kann man sagen, daß der ewig lebt, der in der Gegenwart lebt. (08/07/1916).*

*Ludwig J. J. Wittgenstein (1889-1951)*

*É feliz aquele que não se permite ter temor, nem mesmo da morte.*

*Apenas quem não no Tempo, mas sim no Presente vive, é feliz. (...)*

*Quando se entende por Eternidade, não duração infinita, mas sim atemporalidade, então, pode-se dizer que se vive eternamente, que se vive no Presente.*

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## I. INTRODUÇÃO GERAL

### I. 1. Importância Epidemiológica de *Aedes aegypti*

De acordo com dados da Organização Mundial de Saúde, aproximadamente dois terços da população mundial vive, atualmente, em áreas infestadas por *Aedes aegypti* (PAHO, 1997). Esse vetor é responsável pela transmissão da febre amarela, dengue e dengue hemorrágica. A distribuição mundial de *A. aegypti* e a ocorrência da dengue e febre amarela, de acordo com o “Center for Disease Control and Prevention”, nos Estados Unidos, constam, respectivamente, das Figuras 1 e 2. Quanto à dengue hemorrágica, tem sido constatada com freqüências variáveis, em praticamente todos os países em que ocorre a dengue.

A febre amarela é endêmica em alguns Estados brasileiros e em Países da América do Sul e do continente Africano. Pode ocorrer em três situações distintas: urbana, silvestre e intermediária (ou rural) (WHO, 2001a). Etiologicamente a doença é a mesma e pode matar de 15 a 50% dos indivíduos infectados. A diferença está apenas nos transmissores e no local geográfico de aquisição da infecção. O ciclo natural do vírus da febre amarela envolve espécies de mosquitos pertencentes aos gêneros *Haemagogus* e *Sabethes* e macacos infectados (Monath, 1994). Na área urbana o ser humano faz parte do ciclo e a transmissão se dá, freqüentemente, através do *A. aegypti*. Em áreas fronteiras de mata e atividade agrícola, pode haver uma adaptação do transmissor silvestre ao novo hábitat com a conseqüente possibilidade de transmissão intermediária da febre amarela nesses locais. Em outras circunstâncias, a manutenção do vírus na natureza pode ocorrer

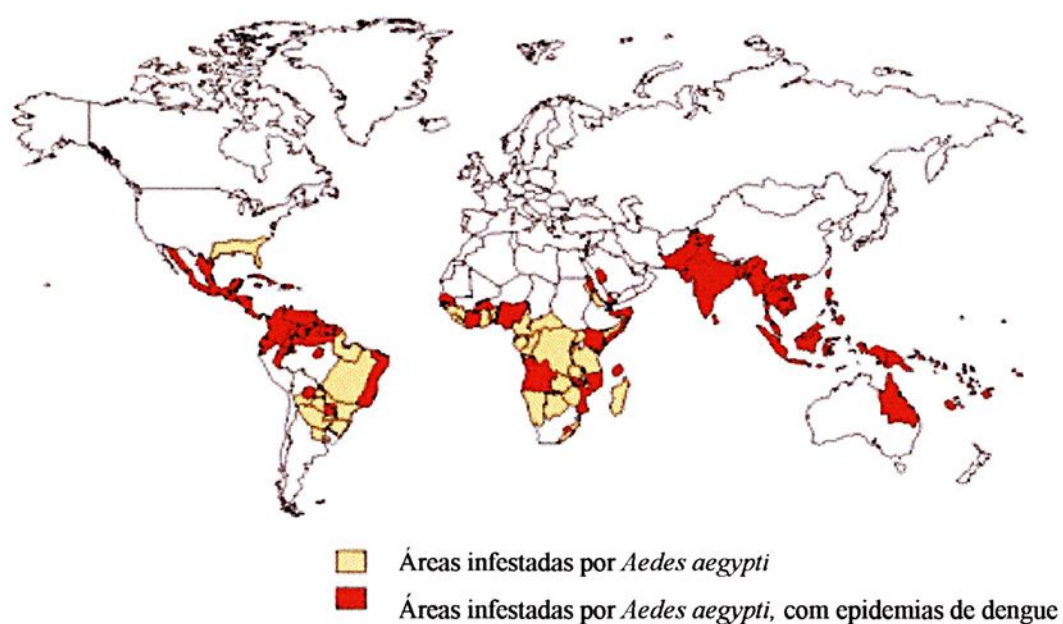


Figura 1. Distribuição mundial do mosquito *Aedes aegypti*, com destaque das áreas com ocorrência de epidemias de dengue no ano de 2000 (Center for Disease Control and Prevention, 2001).

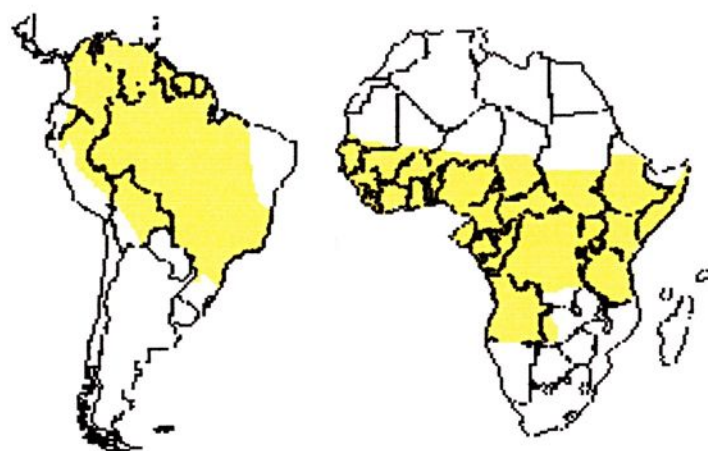


Figura 2. Distribuição mundial das áreas com incidência de febre amarela (modificado de Prata, 2000 e Center for Disease Control and Prevention, 2001).

por meio da transmissão vertical ou transovariana, ou seja, os mosquitos infectados transmitem o vírus por várias gerações (Fontenille *et al.*, 1997). Nas duas últimas décadas, a ocorrência de epidemias e o número de países com a manifestação de febre amarela têm aumentado, embora exista há mais de 60 anos, uma vacina altamente efetiva que confere imunidade contra essa doença por cerca de 10 anos (Prata, 2000).

No Brasil, a erradicação do *A. aegypti* na década de 30, aliada a programas de vacinação, interrompeu a transmissão urbana da febre amarela e também da dengue. Desde a reintrodução do *A. aegypti*, em 1981, passou a existir um evidente risco do retorno da febre amarela urbana e o ressurgimento de epidemias de dengue e sua forma mais grave.

O ressurgimento da dengue e a emergência da dengue hemorrágica tornaram-se, nos últimos anos, um grave problema de saúde pública também no âmbito mundial. A doença é endêmica em mais de 100 Países na África, Américas, leste do Mediterrâneo, sudeste da Ásia e oeste do Pacífico (WHO, 2001b). Existem quatro sorotipos virais que causam a dengue. A forma hemorrágica da doença é atribuída à infecção sequencial por um sorotipo viral diferente do anterior. O Brasil está entre os países em que a situação é preocupante. Segundo dados da Fundação Nacional de Saúde (FUNASA), existem focos da dengue em 20 Estados e em 14 deles ocorrem, simultaneamente, dois dos quatro tipos da doença (Tipos 1 e 2). No início do presente ano, foi registrado o aparecimento de mais um sorotipo da dengue no Brasil, o Tipo 3, o que aumenta o risco, para pessoas que já tiveram dengue, de desenvolver a dengue hemorrágica (Terra *on-line*, 2001).





Segundo a FUNASA, foram registrados 282.541 casos de dengue no País até junho de 2001 – 42.448 a mais que o total do ano passado. As regiões Nordeste e Sudeste apresentaram os maiores índices de notificação. No Estado de São Paulo foram notificados 48.024 casos até agosto de 2001, dos quais cerca de 10.000 ocorreram na região de São José do Rio Preto.

## I. 2. Controle de *Aedes aegypti*

O controle da transmissão do vírus da dengue dá-se essencialmente no âmbito coletivo e exige um esforço de toda a sociedade, em virtude da elevada capacidade de adaptação e transmissão de seu principal vetor. Devido ao fato de que *A. aegypti* se desenvolve principalmente em criadouros artificiais, produzidos pelo homem, a sua eliminação dependente, em grande parte, da educação e conscientização da comunidade. Entretanto, estratégias baseadas na participação da população para esse fim não têm sido muito eficazes, tornando-se necessário o uso de inseticidas.

Em 1996, o Governo Federal criou e passou a executar o “Plano de Erradicação do *Aedes aegypti* - (PEAa)”. No entanto, a análise das dificuldades enfrentadas pelo Plano, na implementação dos seus objetivos, levou à priorização das ações objetivando a redução do dano causado pela dengue (FUNASA, 2001).

A Superintendência de Controle de Endemias (SUCEN) avalia através do índice de Breteau a ameaça de epidemia de dengue, pelo tamanho da população de mosquitos e procede à pulverização com inseticidas nas regiões mais afetadas.

A infestação por *A. aegypti* no Estado de São Paulo teve início na década de 80, paralelamente à reintrodução dessa espécie no Brasil. A partir de 1985,





medidas de controle para esse vetor começaram a ser efetuadas pela SUCEN em conjunto com as prefeituras municipais do Estado (Glasser & Gomes, 2000). Iniciou-se o controle com os inseticidas organofosforados (OPs) temephos (nome comercial Abate®), para larvas do mosquito, e fenitrothion como adulticida de ação residual e também com ação larvicida. Nos anos de 1987 a 1993 o malathion (OP) foi utilizado em pequena quantidade em substituição ao fenitrothion, em períodos de falta desse último no mercado. As nebulizações também vêm sendo realizadas com os inseticidas propoxur (1986 a 1989) e cipermetrina (1989 até o momento), os quais pertencem ao grupo dos carbamatos e piretróides, respectivamente. Os organofosforados continuam sendo utilizados para o controle de larvas (Macoris *et al.*, 1999).

### I. 3. Desenvolvimento de resistência aos inseticidas

O número de espécies de insetos que desenvolveram resistência tem aumentado progressivamente. Os casos de resistência se intensificaram na década de 50, logo após a introdução dos inseticidas organo-sintéticos (Figura 3).

Testes de susceptibilidade, baseados na resposta de mortalidade frente à exposição à dose diagnóstica, foram iniciados pela SUCEN a partir de 1996, para monitorar os efeitos dos inseticidas empregados para o controle do *A. aegypti*. Infelizmente, algumas populações brasileiras, desse vetor, estão manifestando resistência aos inseticidas utilizados. Estudos citogenéticos na população de *A. aegypti* de São José do Rio Preto-SP mostraram a presença de altas frequências de aberrações cromossômicas que poderiam estar relacionadas ao uso freqüente de



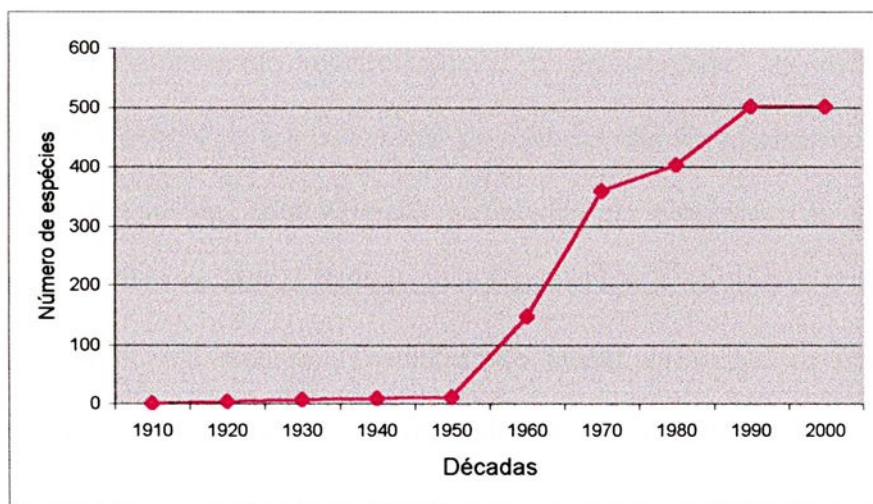


Figura 3. Aumento do número de espécies de artrópodes resistentes a inseticidas, a partir da década de 50 (modificado de Georghiou & Lagunes-Tejeda, 1991).

inseticidas (Lima-Catelani & Bicudo, 1994). De acordo com testes baseados na dose diagnóstica, a população de *A. aegypti* de Goiânia-GO teria apresentado resistência aos inseticidas temephos e fenitrothion (Macoris *et al.* 1995), enquanto que a população de São José do Rio Preto-SP ainda seria susceptível aos mesmos compostos (Macoris *et al.* 1999).

Em programas de monitoramento, o entendimento da evolução da resistência é essencial para a elaboração de medidas efetivas visando ao controle. Por exemplo, uma das características exploradas em programas de manejo da resistência é o fato de que os indivíduos resistentes apresentam, normalmente, um custo adaptativo, ou seja, os resistentes são menos aptos que os susceptíveis quando um produto químico não é utilizado (Georghiou, 1994). O menor valor adaptativo dos indivíduos resistentes pode estar associado a menor fecundidade, maior tempo para o desenvolvimento, menor competitividade para o acasalamento, maior susceptibilidade aos inimigos naturais, etc. (Roush & McKenzie, 1987). Sendo assim, o restabelecimento da susceptibilidade pode ser observado em situações em que um determinado produto não é aplicado por um certo período de tempo. Em situações em que a resistência é instável, isto é, a frequência de alelos resistentes diminui na ausência da pressão seletiva, esta instabilidade pode ser explorada em programas de manejo da resistência com o intuito de preservar indivíduos susceptíveis em uma determinada população. Algumas recomendações dentro desta estratégia incluem a aplicação menos freqüente de inseticidas e manutenção de áreas não tratadas para servir de refúgio aos indivíduos susceptíveis (Georghiou, 1994).





No Brasil, os programas de controle do *Aedes aegypti* têm falhado em alcançar os seus objetivos e como consequência, a dengue e a dengue hemorrágica estão atingindo níveis preocupantes. A educação sanitária visando a participação da comunidade na eliminação do vetor e a busca de formas alternativas de controle são de fundamental importância. O fato de que tem ocorrido desenvolvimento de resistência pelos insetos traz sérias consequências para o homem e o meio ambiente, ocasionando o uso de doses mais elevadas de inseticidas, o uso indevido de misturas e até mesmo substituição de um inseticida por outro, de maior toxicidade. Uma das formas alternativas de controle, que pode ajudar a diminuir o uso indiscriminado de inseticidas organofosforados granulados, é a substituição pela borra do café, que em quantidade adequada é capaz de bloquear o crescimento das larvas do mosquito em criadouros domésticos (Laranja, 2001; Laranja & Bicudo, *in press*).

#### I. 4. Mecanismos de Resistência

As tentativas de controle dos mosquitos têm sido grandemente dificultadas pela sua capacidade reprodutiva e flexibilidade genética elevadas, incluindo a dispersão de genes de resistência a inseticidas nas populações o que pode agravar a disseminação das doenças transmitidas (Besansky & Collins, 1992).

De modo geral, os mecanismos bioquímicos e fisiológicos da resistência, em diferentes organismos, podem ser categorizados como: insensibilidade ao sítio alvo e aumento da desintoxicação metabólica. Estes são causados, em nível molecular, por mutações em ponto na subunidade do receptor GABA, que controla os canais de cloro (para inseticidas ciclodienos); mutações em ponto no



sítio ativo da acetilcolinesterase (AChE) (para inseticidas carbamatos e organofosforados); mutações ligadas geneticamente a canais de sódio (para DDT e inseticidas piretróides); amplificação de genes para esterases (para inseticidas carbamatos e organofosforados); e mutações ainda não caracterizadas em enzimas de desintoxicação, tais como oxidases e glutathione S-transferase - GST (várias classes de inseticidas) (Feyereisen, 1995, Berrada & Fournier, 1997, Hemingway & Ranson, 2000). Em uma população de insetos, geralmente ocorre a combinação de vários mecanismos que resultam na expressão global da resistência. O sistema GST atua, freqüentemente, como um mecanismo de resistência secundário, juntamente com oxidases ou esterases (Hemingway, 2000).

As enzimas responsáveis pela desintoxicação de xenobióticos nos organismos vivos, tais como as oxidases, GSTs e esterases, são representadas por membros de famílias multigênicas. Essas enzimas, geralmente, degradam os inseticidas em moléculas não tóxicas ou menos tóxicas. A desintoxicação aumentada é devida a modificações nas propriedades catalíticas das enzimas, à sua maior produção ou a ambas. A produção aumentada da enzima é em alguns casos devida à amplificação gênica (para esterases) como já mencionado, ou à superexpressão transcricional (para GSTs e oxidases) (Feyereisen, 1995; Berrada & Fournier, 1997).

A maioria dos inseticidas utilizados atualmente estão sujeitos à degradação pelas esterases e dessa maneira, essas enzimas constituem um dos principais mecanismos pelos quais os insetos se tornam resistentes. Em geral, a resistência tem sido relacionada com níveis mais altos de atividade esterásica (Mourya *et al.*, 1993).





As esterases são divididas em quatro classes principais: arilesterases, acetilesterases, carboxilesterases e colinesterases. A base da distinção entre essas classes é a existência ou não de sensibilidade a concentrações diagnósticas de três grupos de inibidores: sulfato de eserina, reagentes sulfidrílicos e organofosforados (Holmes & Masters, 1967). As carboxilesterases e as colinesterases são as enzimas melhor caracterizadas dentro da superfamília  $\alpha\beta$  hidrolases (Russel *et al.*, 1996) e têm sido muito estudadas na avaliação da resistência aos inseticidas organofosforados, carbamatos, piretróides e organoclorados. A resistência baseada em carboxilesterases foi descrita para mais de 30 espécies de insetos de importância médica, veterinária e agrícola (Hemingway & Karunaratne, 1998). Entre os mosquitos, a resistência a inseticidas organofosforados, baseada em esterases, é o mecanismo mais comum. Essas enzimas atuam na desintoxicação, reduzindo a quantidade do produto tóxico, ou seja, elas agem por seqüestro das moléculas do inseticida (Cuany *et al.*, 1993, Callaghan *et al.*, 1998). Nesses casos a resistência pode somente ser obtida quando grandes quantidades da enzima são produzidas (Karunaratne *et al.*, 1993). Entretanto, há casos em que a superprodução não está envolvida. Genes para esterases mutados, produzem enzimas diferentes que são capazes de metabolizar um determinado inseticida com mais eficiência. No inseto *Lucilia cuprina* (Campbell *et al.*, 1998) e no mosquito *Culex tarsalis* (Whyard *et al.*, 1994), carboxilesterases estruturalmente alteradas em populações resistentes, produzem altos níveis de resistência ao malathion.

A resistência a inseticidas envolvendo AchE é normalmente qualitativa. Uma ou mais mutações em ponto no gene *Ace* reduzem a susceptibilidade da



enzima à inibição. A resistência também pode ser devida a modificações quantitativas, embora ainda não encontrada em populações naturais. Altos níveis de AchE no sistema nervoso central, protegem o inseto do envenenamento por organofosforados (Berrada & Founier, 1997).

Em mosquitos, os mecanismos mais frequentes de resistência a organofosforados são as esterases produzidas em grandes quantidades e a AchE insensível (Pasteur & Raymond, 1996). A maior produção de uma única esterase confere resistência por aumentar a hidrólise e o seqüestro do inseticida (Raymond *et al.*, 1998). Esterases altamente ativas são classificadas em A e B com base em sua hidrólise preferencial pelos substratos  $\alpha$  e  $\beta$ -naftil acetatos, respectivamente (Wirth, 1998). Em *Culex*, existe uma alta diversidade de esterases amplificadas em diferentes populações (esterases A1, A2, A3, A4, A5, A8, B1, B2, B3, B4, B5 e B8) (Raymond *et al.*, 1998; Hemingway & Karumaratne, 1998; Qiao *et al.*, 1998, 1999). Os genes para tais esterases espalham-se rapidamente em uma população devido à seleção dos indivíduos resistentes em áreas tratadas (Poirié *et al.*, 1992).

As oxidases também constituem um mecanismo importante pelo qual os insetos se tornam resistentes a inseticidas. Níveis aumentados de oxidases em insetos resistentes resultam de superexpressão e aumento na desintoxicação do inseticida (Carino *et al.*, 1994). Nos insetos essas enzimas catalisam, também, reações envolvidas no crescimento, desenvolvimento e tolerância a toxinas de plantas (Liu & Scott, 1997).

A literatura registra ainda dois mecanismos de resistência, além dos mencionados: a penetração reduzida dos inseticidas em decorrência de alterações



na cutícula dos insetos e a resistência comportamental, na qual os insetos evitam o contato com locais que contenham a substância tóxica (Priester & Georghiou, 1980; Quisenberry *et al.*, 1984; Lockwood *et al.*, 1985; Raymond *et al.*, 1989; Breaud, 1993).

O nível de resistência em populações de insetos vetores é dependente do volume e frequência dos inseticidas aplicados. O rápido desenvolvimento de resistência em mosquitos é favorecido por características que incluem o curto ciclo de vida e a progênie abundante (Georghiou & Taylor, 1977; Hemingway & Ranson, 2000).

### 1. 5. Resistência a inseticidas em *Aedes aegypti*

Vários *loci* conferindo resistência em *A. aegypti* foram mapeados por recombinação simples (Hitchen & Wood, 1974, 1975; Malcolm & Wood, 1982). Dois genes principais foram identificados, um situado no cromossomo 2, conferindo resistência do tipo metabólica (GST), e outro, localizado no cromossomo 3, conferindo resistência não metabólica (Malcolm & Wood, 1982).

Severson *et al.* (1997) descreveram um mapa baseado em marcadores moleculares RFLP (polimorfismo de comprimento de fragmento de restrição) para os 3 sítios alvos dos inseticidas (GABA, canais de sódio e AChE insensível). Os autores confirmaram os resultados obtidos anteriormente, inferindo que o *locus* DDT/piretróides do cromossomo 3 estaria associado com insensibilidade do sítio alvo e que um *locus* adicional, conferindo resistência a DDT, no cromossomo 2, estaria, provavelmente, associado com atividade metabólica aumentada, mediada por GST, ou atividade das oxidases mediadas pelo citocromo P450.





Em *A. aegypti*, estudos de resistência, pela sua importância em saúde pública, ainda são escassos e mostram variações de resultados em diferentes populações analisadas por diferentes autores. Na literatura há indicações quanto ao envolvimento das esterases na resistência, atuando em relação a organofosforados, enquanto a resistência a piretróides estaria relacionada ao mecanismo baseado em oxidases (Kumar *et al.*, 1991).

Na Malásia a atividade de GSTs e carboxilesterases foram determinadas em larvas de *A. aegypti* de duas populações coletadas no campo (Kuala Lumpur e Klang), para verificar a relação entre níveis enzimáticos e resistência aos inseticidas utilizados (DDT, Lindane, Temephos e Carbaryl). Com base em testes de DL<sub>50</sub> as duas populações mostraram-se susceptíveis a todos os inseticidas testados. O nível encontrado de GSTs e carboxilesterases foram baixos, indicando que as larvas ainda não tinham capacidade suficiente para desintoxicar o inseticida utilizado (Shamaan *et al.*, 1993).

Inseticidas organofosforados têm sido utilizados no controle do *A. aegypti* durante os últimos 15 a 20 anos, em várias regiões do mundo (Georghiou *et al.*, 1987). O temephos (Abate<sup>®</sup>), impregnado em grânulos de areia, é aplicado para controle larval em reservatórios hídricos, inclusive os de água potável, especialmente quando sua origem é duvidosa ou é sabidamente contaminada, o que tem favorecido o desenvolvimento de resistência a esse inseticida (Rawlins & Wan, 1995).

A atividade das enzimas acetilcolinesterases, GSTs e esterases foram verificadas em quatro linhagens de *A. aegypti* (Mourya *et al.*, 1993). Nesse trabalho, altos níveis de atividade esterásica em linhagens resistentes comparadas





com susceptíveis, foram considerados como a causa da resistência a OPs. Na resistência a DDT não houve evidências para o envolvimento de GSTs nas linhagens testadas.

Na Índia, estudos durante um surto de dengue em 1992, mostraram que os mosquitos *A. aegypti* eram resistentes ao DDT e em menor nível ao malathion. Análises bioquímicas sugeriram que a resistência ao DDT era devida ao mecanismo baseado no aumento da atividade de GSTs, enquanto a devida ao malathion, a um pequeno aumento na atividade esterásica (Mourya *et al.*, 1994).

Mazzarri & Georghiou (1995) observaram aumento significativo nas esterases de populações naturais da Venezuela quando comparadas com uma linhagem susceptível. Populações de *A. aegypti* do Caribe também apresentaram resistência a vários inseticidas, incluindo temephos, malathion, fenthion e propoxur (Georghiou *et al.*, 1987, Rawlins & Wan, 1995).

No Brasil, poucos estudos têm sido realizados sobre a estrutura genética, baseada em isoenzimas, em populações de *A. aegypti* (Dinardo-Miranda & Contel, 1996, Lima-Catelani, 1996). Entendemos que, em vista da atual situação em que se encontra o País, com altos índices de infestação por esse vetor e de incidência de dengue e risco de epidemias de febre amarela, o conhecimento dos mecanismos envolvidos na resistência, já observada em várias regiões, faz-se necessário.

## I. 6. Uso do fenobarbital em estudos de resistência a inseticidas

O fenobarbital (PB) é uma droga utilizada no tratamento da epilepsia, sendo bastante utilizado em estudos de resistência a inseticidas, por atuar como indutor enzimático. Roedores pré-tratados com essa substância mostraram efeitos menos



tóxicos quando expostos a organofosforados. O aumento na tolerância foi atribuído à indução de hidrolases, carboxilesterases ou oxidases (Nousiainen, 1985; Raftell *et al.*, 1977; Kaneko *et al.*, 1979; Nousiainen & Hänninen, 1981; Clement, 1983; McCracken *et al.*, 1993; Kaliste-Korhonen *et al.*, 1998).

Os valores de  $DL_{50}$ , na intoxicação por organofosforados, aumentaram de 1 a 12 vezes em ratos e de 1 a 3 vezes em camundongos pré-tratados com fenobarbital (Dubois & Kinoshita, 1968). Essa resistência está relacionada ao aumento da atividade de carboxilesterases em 50%, no soro e fígado de camundongo, de colinesterases no plasma (Clement, 1984) e, também, de esterases A no fígado de camundongos (Vitarius *et al.*, 1995).

A toxicidade aguda da somana, um composto organofosforado, é devida, principalmente, à inibição irreversível da acetilcolinesterase. Clement (1983) relatou que sua toxicidade foi significativamente menor em camundongos que tinham sido pré-tratados com fenobarbital. Outros trabalhos ao longo dessa linha mostraram o papel importante de carboxilesterases hepáticas e sanguíneas que também são induzíveis pelo pré-tratamento com fenobarbital na proteção contra a toxicidade da somana (Clement, 1984; Purshottam & Srivastava, 1989).

A inducibilidade das enzimas citocromo P450 tem sido investigada, também, em linhagens resistentes e susceptíveis de *Drosophila melanogaster* e *Musca domestica* (Yu & Terriere, 1977; Hällström *et al.*, 1982; Fuchs *et al.*, 1994; Brun *et al.*, 1996). Esses trabalhos relacionaram o fenobarbital com o grau de atividade da citocromo-oxidase, após tratamento, como via de desintoxicação. Fuchs *et al.* (1994) observaram que linhagens resistentes e susceptíveis de *Drosophila* respondem diferentemente ao tratamento com PB, ou seja, as



primeiras mostraram menor inducibilidade do sistema citocromo P450 que as segundas.

Terriere (1984) propôs que os mesmos genes reguladores podem estar envolvidos na indução pelo PB e na resistência bioquímica. Plapp (1984) sugere a existência de um gene principal, no cromossomo 2 em mosca doméstica, que estaria envolvido na resistência. Outro estudo demonstrou que a indução pelo PB está ligada ao autossomo 2 de mosca doméstica (Liu & Scott, 1997). A transcrição aumentada de CYP6D1, por sua vez, é controlada por fatores localizados nos autossomos 1 e 2, em moscas da linhagem altamente resistente a piretróides, LPR (Learn Pyrethroid Resistant) (Liu & Scott, 1997). A transcrição aumentada de CYP6D1 não é induzida pelo PB na linhagem LPR, mas sim na susceptível. Scott (1999) propôs, então, um modelo para o aumento na transcrição de CYP6D1, induzível por PB. A idéia é que existe um gene no autossomo 2, de moscas susceptíveis, cujo produto é aumentado em resposta ao tratamento com PB e atua para ampliar a transcrição de outros genes. Ainda segundo Scott (1999), se esse gene está sob controle de um elemento regulador (um repressor), e este sofre mutação, pode resultar em expressão constitutiva aumentada, de várias oxidases mediadas pelo citocromo P450. Neste caso, a linhagem pode se tornar resistente.





## II. OBJETIVOS

A busca de informações sobre o desenvolvimento de resistência a inseticidas em populações brasileiras de *Aedes aegypti*, mostra-se importante em virtude dos altos índices de infestação por esse vetor em muitas regiões do País. A abordagem desse objetivo, no presente trabalho, foi realizada pelos seguintes estudos:

1. Análise do padrão de esterases da população de *A. aegypti* de São José do Rio Preto (SJRP) para comparação com estudo prévio realizado por Lima-Catelani (1996), visando detectar possíveis modificações no tempo decorrido entre as análises, nessa população submetida a contínuas aplicações de inseticidas;
2. Análise do padrão de esterases da população de Goiânia (GO), considerada resistente por Macoris *et al.* (1995), com o objetivo de verificar se as modificações ocorridas em SJRP estão presentes nessa linhagem;
3. Pré-tratamento com fenobarbital de amostras das populações de SJRP e GO, seguido de testes das amostras pré-tratadas e não pré-tratadas (controle) ao organofosforado temephos, para verificar se essa substância induz tolerância em *A. aegypti*;
4. Tendo em vista que os resultados do item 3 foram positivos, análise do padrão de esterases de indivíduos pré-tratados com fenobarbital e comparação com o dos não pré-tratados, a fim de verificar possíveis alterações relacionadas com a tolerância;
5. Também, pelos resultados positivos do item 3, realizou-se tratamento dos mosquitos com butóxido de piperonila para melhor conhecimento dos mecanismos envolvidos na resistência.





### III. ARTIGO 1

## MULTIPLE CHANGES IN ESTERASE PATTERNS OF *Aedes aegypti* RESISTANT TO INSECTICIDES

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*Key Words:* *Aedes aegypti*, esterases, insecticide resistance

## Resumo

Larvas (L4), pupas e adultos de *Aedes aegypti* de São José do Rio Preto-SP (SJRP) e de Goiânia-GO (GO, considerada resistente a inseticidas), foram analisadas quanto ao padrão de esterases, que são enzimas associadas com resistência em vários organismos. Os dados obtidos para SJRP foram comparados com os obtidos há oito anos, para a mesma população, e também àqueles obtidos para GO, com o objetivo de detectar possíveis alterações relacionadas com resistência. A observação de quatro novas bandas (colinesterases) em SJRP e GO (em frequências maiores em GO), indica de forma consistente, que essas esterases estão envolvidas no desenvolvimento de resistência na primeira população. Várias outras similaridades foram observadas na análise atual, entre SJRP e GO assim como diferenças entre a análise prévia e a atual para SJRP. As modificações no padrão de esterases incluíram seleção (modificações na frequência e tipo de bandas) e regulação diferencial (ativação ou repressão) durante o desenvolvimento.

## Abstract

Brazilian samples of *Aedes aegypti* from São José do Rio Preto (SJRP) and from Goiânia (GO, considered insecticide resistant) were studied as to the esterases which are enzymes associated with insecticide resistance in several organisms. The present data on SJRP mosquitoes were compared with data obtained eight years ago for the same population and with those obtained for GO, aiming to detect possible changes on esterase patterns that could be related to insecticide resistance. The finding of new bands in the present population from SJRP and in GO (with higher frequencies in GO), strongly indicates that they are involved with resistance in progress in the first locality. Several other similarities between the present SJRP and GO populations and differences between them and SJRP in the previous analysis were observed. The multiple changes in the esterase patterns were selection (modifying band frequency and type) and differential gene activation (turning on and off) during development.



## Introduction

That the esterase activity and the insecticide resistance in insects are related to each other is already very well known (Mouchès *et al.*, 1987; Raymond *et al.*, 1989; Hemingway & Karunaratne, 1998). This has been shown in several groups of those organisms, including the Australian sheep blowfly, *Lucilia cuprina* (Smyth *et al.*, 1994; Campbell *et al.*, 1997), the housefly, *Musca domestica* (Claudianos *et al.*, 1999), the aphid, *Myzus persicae* (Field & Devonshire, 1998; Field *et al.*, 1999; Blackman *et al.*, 1999) and the mosquitoes *Culex* (Guillemaud *et al.*, 1997; Hemingway & Karumaratne, 1998; Qiao *et al.*, 1999) and *Anopheles* (Penilla *et al.*, 1998; Brogdon *et al.*, 1999).

Among the four esterase groups recognized on the basis of their susceptibility to diagnostic concentrations of specific inhibitors (Holmes & Masters, 1967; Oakeshott *et al.*, 1993), two have been associated with insecticide resistance, carboxylesterases and cholinesterases. The carboxylesterases act in the insecticide degradation. In most cases, the resistance that they provide arises from gene amplification increasing the amount of the respective gene product, that is, the enzyme (Pasteur *et al.*, 1981; Raymond *et al.*, 1989; Mouchès *et al.*, 1990; Poirié *et al.*, 1992). The resistance due to the cholinesterases arises mainly from mutated genes whose products become insensitive to the inhibition by insecticides (Smissaert, 1964; Bourguet *et al.*, 1996; Vaughan *et al.*, 1997).

*Aedes aegypti*, important vector of dengue, dengue hemorrhagic fever (DHF) and yellow fever, common in temperate and tropical countries, has also demonstrated the ability to develop resistance to many insecticides including



organochlorine, organophosphate, carbamate and pyrethroid (Georghiou *et al.*, 1987; Rawlins & Wan, 1995). Some data has already indicated that the resistance development to organophosphate in this vector is dependent on increased esterase activity levels (Mourya *et al.*, 1993; Mazzarri & Georghiou, 1995; Vaughan *et al.*, 1998). Mazzarri & Georghiou (1995) and Vaughan *et al.* (1998) did not find insensitive acetylcholinesterase involved in organophosphate or carbamate resistance, while for pyrethroid resistance different populations showed or not involvement of oxidase enzymes (Kumar *et al.*, 1991; Mazzarri & Georghiou, 1995).

Brazil is among the countries that were reinfested by those mosquitoes and thus is under the risk of the manifestation of the diseases that they transmit. In the present work the esterase patterns of *A. aegypti* from São José do Rio Preto, SP, Brazil, were analyzed. Approximately eight years before, Lima-Catelani (1996) had described for the first time the isozyme system of the same population. During the elapsed time, pulverization with insecticides (mainly pyrethroids) for the adult mosquito control and 1% temephos sand granules (organophosphate) for larval control in breeding-sites (tires and others) have been frequently applied (Bracco *et al.*, 1999). Although Macoris *et al.* (1999) still considered the population of mosquitoes from São José do Rio Preto susceptible, in this new study we intended to detect possible changes in the esterase patterns of the mosquitoes which occurred during this time that could be related to the presence of those insecticides in the environment. As a basis for comparison, the esterase patterns of another *A. aegypti* population, from Goiânia, which was considered resistant in tests performed by the same authors (Macoris *et al.*, 1995) were also

analyzed. Data obtained gave important information related to the state of resistance of *A. aegypti* de São José do Rio Preto compared to that from Goiânia.



## Materials and methods

*Aedes aegypti* from São José do Rio Preto-SP (SJRP) and Goiânia (GO) were analyzed. The examined mosquitoes from SJRP were collected during the study from tires and other artificial containers by SUCEN (Superintendência de Controle de Endemias do Estado de São Paulo) and studied in the first generation produced in the Vector Laboratory, IBILCE/UNESP. The mosquitoes from Goiânia were supplied twice during the experiments by Dr. Ionizete Garcia da Silva from the Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, GO; eggs from recently collected mosquitoes were furnished each time.

Following the same techniques used by Lima-Catelani (1996), esterase patterns were analyzed in 8% polyacrylamide gels (PAGE) (Laemmli, 1970). Fourth-instar larvae, pupae and adult males and females were individually squashed in 25  $\mu$ l of the buffer solution (1.5M Tris-HCl, pH8.8, plus 10% glycerol) for each sample preparation. The supernatant (10 $\mu$ l) was then used for each sample. Electrophoresis was performed for 2hr. at  $\sim$ 25°C using a constant 200V. The running buffer used was 0.1M Tris-glycine at pH8.3.

Esterases were identified in the gels as described by Johnson *et al.* (1966) and Steiner & Johnson (1973), including preincubation (45min.) at room temperature ( $\sim$ 25°C) in 50 ml 0.1M sodium phosphate at pH 6.2, and staining reaction in the dark (1hr.) with a solution containing both  $\alpha$ - and  $\beta$ -naphthyl acetates as substrates (30mg of  $\alpha$ - and 15mg of  $\beta$ -naphthyl acetate), 60mg of fast blue and 5ml of N-propanol in 50ml of the sodium phosphate solution. After this



treatment, the esterase bands show the following characteristic colors: black (for those that hydrolyze  $\alpha$ -naphthyl acetate), red (for those that hydrolyze  $\beta$ -naphthyl acetate) and magenta (for those that hydrolyze both  $\alpha$ - and  $\beta$ -naphthyl acetates). On the basis of these results they were named  $\alpha$ -esterases,  $\beta$ -esterases and  $\alpha\beta$ -esterases, respectively.

The gels were dried at room temperature by using gelatin and cellophane wound slab gels in an embroidering hoop, as described by Ceron *et al.* (1992). The esterase bands in gels were numbered following Lima-Catelani (1996), from the less anodic to the more anodic end of the gels.

For biochemical characterization of the esterases, five inhibitors were individually used, as follows: 0.4mM solution of malathion (organophosphate-OP), 5mM solution of sodium fluoride (NaF) and 0.1mM solutions of eserine sulfate (carbamate) or *p*-chloromercuribenzoate (pCMB) or phenylmethylsulphonyl fluoride (PMSF). Each inhibitor was used in both the pre-incubation and the stain solutions. Gels containing the same samples were used as controls. On the basis of these tests, the esterases corresponding to the bands are classified as carboxylesterases (those inhibited by malathion), cholinesterases (inhibited by both malathion and eserine sulfate), arylesterases (inhibited by pCMB) and acetylersterases (those that are not affected by any of these inhibitors) (Holmes & Masters, 1967; Oakeshott *et al.*, 1993)

Similarity of the examined populations (SJRP and GO, in the present study) and SJRP (Lima-Catelani, 1996) was estimated by using shared and exclusive bands (1 for presence, 0 for absence of the band) and the band frequencies. In these analyses, larvae, pupae and adults were considered together



or larvae were considered separately. The Single Linkage was used for cluster analysis (Manzato, 1983; Johnson & Wichern, 1992) and the Pearson Correlation was used for similarity measure (Mardia *et al.*, 1979).



## Results

The esterase patterns of *Aedes aegypti* larvae, pupae and adults from SJRP and GO observed in this work, are presented in Figures 1 and 2. Among the total of 20 esterase bands, nine hydrolyzed the substrate  $\alpha$ -naphthyl acetate (EST-1 to EST-7, EST-11 and EST-16) and thus they were classified as  $\alpha$ -esterases; three hydrolyzed the  $\beta$ -naphthyl acetate (EST-19 to EST-21) and were classified as  $\beta$ -esterases; and eight hydrolyzed both  $\alpha$  and  $\beta$  -naphthyl acetates (EST-A to EST-D, EST-9, EST-12 to EST-14) being classified as  $\alpha\beta$ -esterases. Among the 20 bands, 19 were detected in SJRP (EST-A to EST-D; EST-1 to EST-7; EST-9; EST-11 to EST-14; EST-19 to EST-21) and 18 in GO (EST-6 and the others found in SJRP, except EST-11 and EST-21). The frequencies of esterase bands observed in two localities are presented in Table 1.

A comparison between the esterase patterns observed in the present study in mosquitoes from SJRP and those observed by Lima-Catelani (1996) showed four new bands ( $\alpha\beta$ -esterases EST-A to EST-D) located in the less negatively charged region of the gels were detected in SJRP as well as in GO, in the present study. They were not observed by the previous author in a total of 162 larvae, 120 pupae and 119 adults analyzed from SJRP. These new bands were now detected in larvae, pupae and adult males with frequencies that varied from 1.5% to 13.8%, but in GO they showed frequencies higher than those, mainly in larvae and pupae (for example, 87.3% and 64.6%, respectively, for one of these bands-EST-C).

On the other hand, three bands described by Lima-Catelani (1996) were not found in this study. They were named by that author EST-8, EST-15 and EST-

18. In that study, the first band had a frequency of 24.7% in fourth-instar larvae, the only stage in which it was observed. The second band was found in pupae and adults, showing a low frequency in pupae (2.5%) and adult males (8.6%) but in adult females the frequency was higher (23%); EST-18 was found in low frequency (1.8%).

A fourth band (EST-16) detected by Lima-Catelani (1996) in all stages, with a frequency of 55.8%, in pupae, and frequencies that varied from 8.2% to 15.5% in the other stages, was not found in SJRP in the present study. However, this band appeared with a frequency of 4.8% in pupae from GO.

Another difference is related with the presence of the bands EST-2 to EST-6 and EST-20. This last band was detected by Lima-Catelani (1996) in fourth-instar larvae with a frequency of 82.7% and in pupae with a frequency of 10%; in the present study it was absent in pupae, but remained with a high frequency in larvae, both in SJRP and GO (about 60%). The bands EST-3 to EST-6 were detected by Lima-Catelani exclusively in larvae while in the present study they were also found in other stages with frequencies that varied from 1.6% to 29%, in both populations. For the band EST-2, the opposite occurred. Lima-Catelani (1996) found it in all stages varying in frequency from 2.5% to 34.5% (6.8% in larvae) while in the present study it was exclusively found in larvae, in both SJRP and GO (33.8% and 36.5%, respectively).

Results from  $\chi^2$  statistical analysis for the frequencies of bands observed in both studies from SJRP are shown in Table 2. For larvae, the frequency of seven bands (EST-3 to EST-5, EST-7, EST-12, EST-14 and EST-20) decreased and that

of one band increased (EST-2) significantly. In pupae, the frequency of band EST-12 decreased and that of band EST-7 increased significantly.

In the adult females only band EST-14 presented frequency decrease. In the adult males, the frequency of bands EST-12 to EST-14 decreased and that of band EST-7 increased. Bands EST-12 and EST-14 showed significant reduction in every developmental stage, except EST-14 in pupae and EST-12 in adult females.

The bands that presented differences in frequency between SJRP (the present analysis) and GO populations are also in Table 2. Six bands in larvae (EST-B to EST-D, EST-4, EST-5 and EST-9), four bands in pupae (new bands EST-B, EST-C, EST-D and band EST-13), two bands in adult females (EST-D and EST-14) and one in adult males (EST-7) presented frequencies greater in the GO population than in SJRP. On the other hand, two bands in larvae (EST-6 and EST-7), one in pupae (EST-5) and one in adult females (EST-3) presented frequencies greater in the SJRP population.

Inhibition patterns obtained in both populations are in the Table 3. Bands EST-1 and EST-2 were completely inhibited by PMSF and malathion and moderately inhibited by NaF. The esterases EST-3 to EST-7 were completely inhibited by malathion and slightly inhibited by eserine (moderately in EST-7), PMSF and NaF. EST-12 to EST-14 were totally inhibited by eserine and malathion and strongly inhibited by PMSF (moderately in EST-13). EST-19 to EST-21 were totally inhibited by NaF and malathion and partially affected by PMSF. The new esterases EST-A to EST-D were affected by all inhibitors tested, but more strongly by eserine and malathion. EST-9 was tested with all inhibitors



except malathion, showing weak inhibition with PMSF and NaF and no inhibition with eserine and pCMB.

These results suggested that the bands may be classified as follows: - cholinesterases: EST-A to EST-D, EST-12 to EST-14; - carboxylesterases: EST-1 to EST-7, EST-19 to EST-21. The esterase EST-9 could not be classified on the basis of these results.

Figure 3 shows the result of cluster analysis, including the similarity values for every comparison. In every case, except esterase band frequency considering only larvae, SJRP2 (the present SJRP population) and GO were closer to each other than to SJRP1 population in the previous analysis. Similarity levels considering band frequencies were higher than those considering presence/absence.



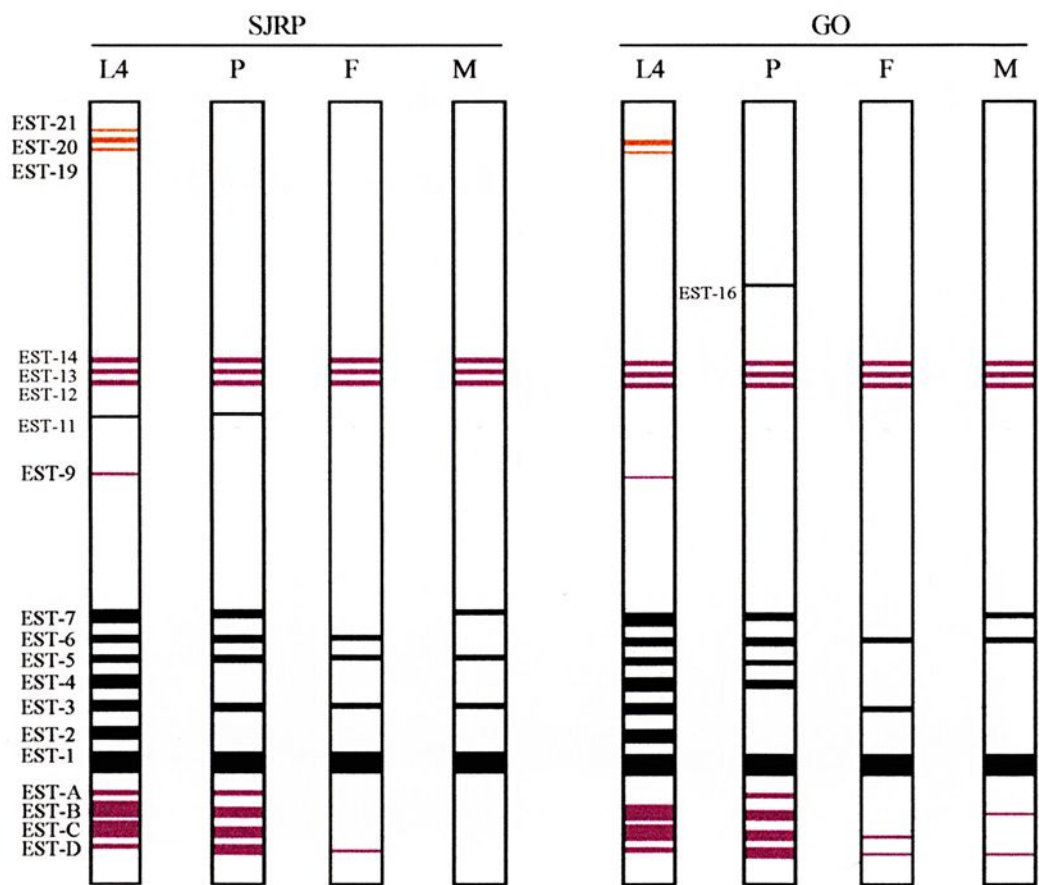


Figure 1. Patterns of esterase bands of *Aedes aegypti* observed in the present study, in São José do Rio Preto (SJRP) and Goiânia (GO).  $\alpha$ -esterases ( — ),  $\beta$ -esterases ( — ) e  $\alpha\beta$ -esterases ( — ).

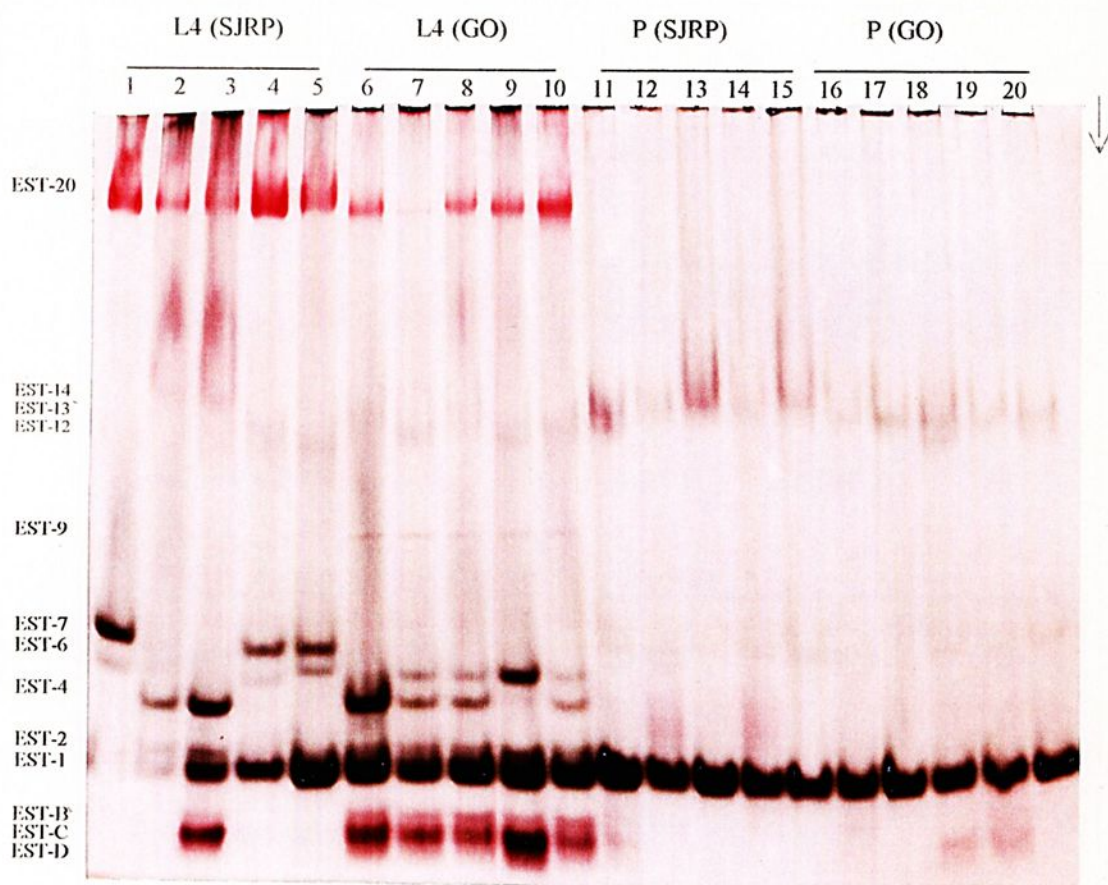


Figure 2. Esterase patterns in polyacrylamide gel from São José do Rio Preto (SJRP) and Goiânia (GO) populations of *Aedes aegypti*. L4= larvae, P= pupae. Observe new bands (EST-B, EST-C and EST-D) in the less negatively charged region of the gel.

|                          |       |       |       |
|--------------------------|-------|-------|-------|
|                          |       | 91.38 |       |
|                          |       |       | 92.52 |
| Frequency L, P, A        | SJRP1 | SJRP2 | GO    |
|                          |       | 86.59 |       |
|                          | 93.66 |       |       |
| Frequency L              | SJRP1 | SJRP2 | GO    |
|                          |       | 89.46 |       |
|                          |       | 94.58 |       |
| Frequency P, A           | SJRP1 | SJRP2 | GO    |
|                          |       | 65.32 |       |
|                          |       | 84.82 |       |
| Presence/Absence L, P, A | SJRP1 | SJRP2 | GO    |
|                          |       | 62.63 |       |
|                          |       | 84.68 |       |
| Presence/Absence L       | SJRP1 | SJRP2 | GO    |
|                          |       | 63.55 |       |
|                          |       | 83.58 |       |
| Presence/Absence P, A    | SJRP1 | SJRP2 | GO    |

Figure 3. Proximity based on esterase patterns between the samples analyzed in the present study (SJRP2 and GO) and SJRP sample previously analyzed (SJRP1, Lima-Catelani, 1996). Similarity values are indicated on the lines. L= larvae; P= pupae; A= adult.



Table 1. Frequency of the mosquitoes carrying esterase bands in *Aedes aegypti* populations from São José do Rio Preto (SJRP) and Goiânia (GO) in each developmental stage. L4= fourth instar larvae; P= pupae; F= adult females; M= adult males; N= number of analyzed mosquitoes.

|        | SJRP |      |      |      | GO   |      |      |      |
|--------|------|------|------|------|------|------|------|------|
|        | L4   | P    | F    | M    | L4   | P    | F    | M    |
| N      | 65   | 65   | 65   | 65   | 63   | 62   | 64   | 64   |
| Bands  |      |      |      |      |      |      |      |      |
| EST-21 | 6.1  | -    | -    | -    | -    | -    | -    | -    |
| EST-20 | 61.5 | -    | -    | -    | 63.5 | -    | -    | -    |
| EST-19 | 6.1  | -    | -    | -    | 9.5  | -    | -    | -    |
| EST-16 | -    | -    | -    | -    | -    | 4.8  | -    | -    |
| EST-14 | 23.1 | 44.6 | 24.6 | 26.1 | 20.6 | 43.5 | 45.3 | 17.2 |
| EST-13 | 44.6 | 47.7 | 49.2 | 40.0 | 50.8 | 66.1 | 48.4 | 56.2 |
| EST-12 | 23.1 | 16.9 | 12.3 | 13.8 | 27.0 | 19.3 | 9.4  | 17.2 |
| EST-11 | 3.1  | 3.1  | -    | -    | -    | -    | -    | -    |
| EST-9  | 52.3 | -    | -    | -    | 77.8 | -    | -    | -    |
| EST-7  | 32.3 | 63.1 | -    | 30.8 | 12.7 | 53.2 | -    | 50.0 |
| EST-6  | 40.0 | 20.0 | 4.6  | -    | 15.9 | 29.0 | 1.6  | 1.6  |
| EST-5  | 33.8 | 10.7 | 3.1  | 4.6  | 55.5 | 1.6  | -    | -    |
| EST-4  | 69.2 | -    | -    | -    | 88.9 | 6.4  | -    | -    |
| EST-3  | 44.6 | 15.4 | 16.9 | 13.8 | 42.9 | -    | 1.6  | -    |
| EST-2  | 33.8 | -    | -    | -    | 36.5 | -    | -    | -    |
| EST-1  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  |
| EST-A  | 1.53 | 4.6  | -    | -    | -    | 14.5 | -    | -    |
| EST-B  | 12.3 | 4.6  | -    | -    | 76.2 | 43.5 | -    | 1.56 |
| EST-C  | 13.8 | 7.7  | -    | -    | 87.3 | 64.5 | 3.1  | -    |
| EST-D  | 4.6  | 6.1  | 1.5  | -    | 44.4 | 56.4 | 14.1 | 9.4  |

- indicates absence of bands in the analyzed samples

Table 2. Significant differences in the comparison of band frequencies between Lima-Catelani's study (SJRP-1993-95) and the present study (SJRP-1999-2001) and between the last one with Goiânia population (GO-1999-2001), in each analyzed stage. (\* $p < 0.05$ ; \*\* $p < 0.01$ ). > = increase in band frequency; < = decrease in band frequency.

| SJRP- 1999-2001 x SJRP- 1993-95 |             |             |             | GO- 1999-2001 x SJRP- 1999-2001 |            |            |           |
|---------------------------------|-------------|-------------|-------------|---------------------------------|------------|------------|-----------|
| L4xL4                           | PxP         | FxF         | MxM         | L4xL4                           | PxP        | FxF        | MxM       |
| EST-20 < **                     |             |             |             |                                 |            |            |           |
| EST-14 < **                     |             | EST-14 < ** | EST-14 < *  |                                 |            | EST-14 > * |           |
|                                 |             |             | EST-13 < ** |                                 | EST-13 > * |            |           |
| EST-12 < **                     | EST-12 < ** |             | EST-12 < ** |                                 |            |            |           |
|                                 |             |             |             | EST-9 > **                      |            |            |           |
| EST-7 < **                      | EST-7 > **  |             | EST-7 > *   | EST-7 < **                      |            |            | EST-7 > * |
|                                 |             |             |             | EST-6 < **                      |            |            |           |
| EST-5 < **                      |             |             |             | EST-5 > *                       | EST-5 < *  |            |           |
| EST-4 < **                      |             |             |             | EST-4 > **                      |            |            |           |
| EST-3 < **                      |             |             |             |                                 |            | EST-3 < ** |           |
| EST-2 > **                      |             |             |             |                                 |            |            |           |
|                                 |             |             |             | EST-B > **                      | EST-B > ** |            |           |
|                                 |             |             |             | EST-C > **                      | EST-C > ** |            |           |
|                                 |             |             |             | EST-D > **                      | EST-D > ** | EST-D > ** |           |

Table 3. Inhibitor effect on the esterase bands of *Aedes aegypti* from São José do Rio Preto and Goiânia (-: no inhibition; +, ++, +++, ++++: increasingly greater inhibition).

| Inhibitor | Concentration | Esterase bands |         |      |      |   |      |      |      |       |
|-----------|---------------|----------------|---------|------|------|---|------|------|------|-------|
|           |               | A-D            | 1 and 2 | 3-6  | 7    | 9 | 12   | 13   | 14   | 19-21 |
| Malathion | 0.4mM         | ++++           | ++++    | ++++ | ++++ | ? | ++++ | ++++ | ++++ | ++++  |
| Eserine   | 1mM           | ++++           | -       | +    | ++   | - | ++++ | ++++ | ++++ | -     |
| PMSF      | 1mM           | +++            | ++++    | +    | +    | + | +++  | ++   | +++  | ++    |
| pCMB      | 1mM           | ++             | -       | -    | -    | - | -    | -    | -    | -     |
| NaF       | 5mM           | ++             | ++      | +    | +    | + | -    | -    | -    | ++++  |

## Discussion

Approximately eight years have passed by since Lima-Catelani (1996) performed her study on esterase patterns of *Aedes aegypti* from SJRP. During the elapsed time, the *A. aegypti* population size was monitored by the Superintendência de Controle de Endemias do Estado de São Paulo (SUCEN) that used frequent insecticide application for its control. The insecticides used were mainly pyrethroids for adult control, but temephos (Abate<sup>®</sup>, organophosphate) was also used for larval stages. As a consequence, resistance to these insecticides might have developed during this time.

Because esterase changes are one of the major mechanisms by which the insects become resistant, a new analysis of the esterase patterns of the *A. aegypti* from SJRP and a comparison of these data with those in the previous analysis (Lima-Catelani, 1996) could reveal differences related to that mechanism. Besides this, the analysis of a sample considered resistant, could give an important basis for evaluation of the involvement of the observed changes in resistance. The population from GO, considered resistant by Macoris *et al.* (1995), was chosen for this purpose.

Qualitative and quantitative differences on esterase patterns were found in the comparisons of the two SJRP analyses (1993-95 and 1999-2001) and in the comparison with GO data.

In the present analysis of SJRP samples, four new  $\alpha\beta$ -esterases bands, classified as cholinesterases, located in the less negatively charged region of the gels were observed. The presence of these new bands also in the population from



GO (considered resistant), with higher frequencies than in the present population from SJRP, strongly indicates that they are related to esterases involved with resistance and that mosquitoes from SJRP carrying the genes correspondent to these bands are being selected.

It is also interesting that these new bands occur with higher frequencies and with greater activity (the bands are thicker and more intensively stained) in larvae than in pupae and adults. These observations are valid for the two populations presently studied, SJRP and GO. As we mentioned, the larval stage is the one submitted to the control with the temephos insecticide. Then, it seems reasonable to suppose that, in this case, gene selection and activation are occurring mainly at the larval stage.

In the present analysis of SJRP population, band EST-2, classified as a carboxylesterase, significantly increased its frequency in the larval stage (6.8% to 33.8%) and was not detected in the other stages in which it was observed by Lima-Catelani (1996). The same was observed in GO. This band was also considered by Lima-Catelani (1996) as possibly involved with resistance. The present results reinforce this idea.

Instead of increasing, other bands showed a reduction of frequency in larval stage, in the comparisons of both SJRP analyses. They are EST-3 to EST-5, EST-7, EST-12, EST-14 and EST-20. EST-12 and EST-14 were classified as cholinesterases while EST-3 to EST-5, EST-7 and EST-20, as carboxylesterases.

Some bands showed reduction of frequency in the larval stage and a process of band (gene) activation in other developmental stages (for example bands EST-3 and EST-5).

Other differences between the two sets of data from SJRP involved bands that were found by Lima-Catelani (1996) and were not found in the present study. Of course, bands found previously with low frequencies might be not found now by random, but if bands with high frequencies in the previous study are not found now, this may be considered as indicative of a true change. This is the case of EST-8, EST-15 and EST-16, that in the previous analysis had shown 24.7% in larvae, 23% in adult females and 55.8% in pupae, respectively. These bands were not classified biochemically by Lima-Catelani (1996).

The observation of a frequency decrease of some bands and frequency increase of others, or even the absence of some bands previously detected, might suggest a substitution of alleles under the environmental pressure, in which the insecticides are certainly an important component.

Several of these changes observed in the present SJRP population make it more similar to GO population, sometimes suggesting that the pattern of SJRP relative to them is underway to become more similar to the GO pattern. This is the case of EST-7 (apparently in the process of increasing in pupae and in adult males), EST-12 and EST-20 (with a similar pattern in both populations) and EST-14 (with a similar pattern in larval stage). The extended presence of bands EST-3 to EST-6 to other developmental stages was also observed in GO population. The similarity indices confirmed these individual observations, showing that in most comparisons between the esterase patterns observed in the present SJRP population is closer to GO than to the previous SJRP population. These similarities reinforce the idea that in the present SJRP population, evolution of insecticide resistance is occurring.



In the present stage of the study we can not affirm that the esterase changes, occurred in the course of time, between both analyses of *A. aegypti* from SJRP, are due to alterations in the resistance level. However, the observations strongly support this idea. Besides the similarities observed between the present SJRP population and GO, esterases that showed alterations between both SJRP analyses were mostly classified as cholinesterases or carboxylesterases, as mentioned, considered widely involved with insecticide resistance, in several organisms. In addition, bioassays conducted in our Lab, with insecticides, indicated that SJRP mosquitoes are indeed resistant to some insecticides.

Thus the present study shows that multiple changes in the esterase patterns, including selection (modifying band frequency and type) and differential gene activation (turning on and off) during development are occurring in GO and SJRP, affecting several esterase genes. The observations suggest that the development of resistance may be rather due to the sum of several changes in different esterase loci than due to change in the activity of a single locus.

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#### IV. ARTIGO 2

### **HARMFUL EFFECTS OF PHENOBARBITAL IN THE DEVELOPMENT AND LONGEVITY OF *Aedes aegypti***

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**Key Words:** *Aedes aegypti*, phenobarbital, development



## Resumo

Os efeitos biológicos do fenobarbital (PB) foram analisados em *Aedes aegypti*, incluindo porcentagem de eclosão, tempo de desenvolvimento, mortalidade nos estádios de larva e pupa e longevidade dos adultos. Foram utilizadas amostras de duas populações brasileiras, uma de São José do Rio Preto-SP e outra de Goiânia-GO. O tratamento contínuo com PB foi realizado colocando-se os ovos para eclodir em solução contendo 0,2mg/ml de PB, na qual, larvas e pupas permaneceram até completarem o desenvolvimento. O tratamento com PB causou atraso no desenvolvimento e diminuição na longevidade dos adultos. Efeitos drásticos foram também observados no estágio de pupa, em que 30% dos imagos não conseguiram emergir. Alguns deles foram cuidadosamente retirados do casulo, com a ajuda de um pincel fino, mas a maioria dos imagos tratados (aqueles que emergiram normalmente e os que foram retirados do casulo) morreram em poucas horas, ou um dia após deixarem o casulo. Os efeitos são indicativos de que importantes mudanças ocorreram durante o desenvolvimento de *A. aegypti* em resposta ao PB, provavelmente afetando o sistema nervoso, reforçando resultados previamente obtidos em outros organismos.





## Abstract

The biological effects of treatment with phenobarbital (PB), including percentage of egg eclosion, developmental time, mortality in larval and pupal stages and adult longevity were analysed in *Aedes aegypti*. Two Brazilian strains were used: one from São José do Rio Preto, SP and the other from Goiânia, GO. The continuous treatment with PB was performed by putting eggs to eclode in 0.2mg/ml of PB in water solution where they remained till complete development. The treatment produced a delay in the developmental time and decrease in adult longevity. Drastic effects were also observed at the pupal stage, where about 30% of imagoes could not emerge. Some of them were carefully removed from pupal case with a fine brush, but most of both kinds of treated imagoes (those normally emerged and those helped to emerge) died within few hours or one day, after leaving the pupal cases. The effects are indicative that important changes occurred during *A. aegypti* development in response to the PB action, probably affecting the nervous system, thus reinforcing results previously obtained in other organisms.



## Introduction

Phenobarbital (PB) was introduced in 1912 in the treatment of epilepsy. Its effectiveness, low toxicity and low cost turned that drug into an important agent in the treatment of this illness, being administered for a long period to adults and children (Sudha *et al.*, 1996). Experimental studies involving the use of PB, in humans, are difficult due to the interference of factors such as type, severity and frequency of the epileptic attacks as well as the number of drugs administered simultaneously (O'Neill *et al.*, 1977; Trimble and Thompson, 1981). Because of this, the studies with animals have been considered important, contributing significantly to the knowledge of PB effects (Sudha *et al.*, 1996).

Conflicting results have been shown in different studies using PB administration in humans relative to its side effects. Normal adult volunteers and patients with epilepsy, to whom PB was administered, showed low performance in tests of concentration and in tasks requiring short-term memory (Hutt *et al.*, 1968; Macleod *et al.*, 1978). Children treated daily, did not show lower I.Q., but the long treatment caused a transient deterioration of the psychomotor function (Hutt *et al.*, 1968; Hellström and Barlach-Christoffersen, 1980; Wolf *et al.*, 1981).

Studies in mice and rats have shown that the impaired effects are dependent on the doses administered. Disturbance of the locomotion activity is among these negative effects. Stimulating effect in low doses and depressant effect in high doses were observed (Middaugh *et al.*, 1981; File and Wilks, 1990). The administration of high doses of PB also showed effects on mouse learning and memory (Mondadori and Classen, 1984; Rostock and Siegemund, 1993).

However, in therapeutic doses, those effects were not observed (Sudha *et al.*, 1996; Brow-Croyts *et al.*, 2000). An increase in the activity of AChE in the hippocampus was observed in the group of mice treated with high doses of PB (75mg/Kg) (Sudha *et al.*, 1996).

Cell division also seems to be affected by PB. In culture of mouse neurones, the number of cells was reduced to 50% in the treatment with 30 µg/ml of PB (Loegering and Johnson, 1994). The authors suggested that PB interferes with normal interaction between the neurones and glia, necessary for survival of neurones, proliferation of Schwann cells and dendrites induction. PB can still cause hepatic lesions in mice and rats, increasing the DNA synthesis and cell proliferation and decreasing the apoptosis rate (Kolaja *et al.*, 1996). Other studies, however, indicated that the presence of PB induces apoptosis (Chiao *et al.*, 1995, Osanai *et al.*, 1997, Ikonomidou *et al.*, 2000).

PB is also involved in the induction of the cytochrome P450 synthesis. High levels of this enzyme were observed in *Drosophila melanogaster* (Hällström *et al.*, 1982; Brun *et al.*, 1996), in chicken (Lapadula *et al.*, 1984) and in housefly (Yu and Terriere, 1977) submitted to PB treatment. The high synthesis of that enzyme was considered a detoxification mechanism induced by PB.

PB is also involved with insecticide susceptibility. In rodents previously treated with this substance, the use of organophosphate (OPs) showed less toxic effects. This lower toxicity was attributed to the induction, of hydrolases, carboxylesterases or hepatic monooxygenases included in the cytochrome P450 superfamily caused by PB pretreatment (Raftell *et al.*, 1977; Kaneko *et al.*,

1979; Clement, 1983; Kaur and Ali, 1983; Nousiainen, 1985; McCracken *et al.*, 1993; Kaliste-Korhonen *et al.*, 1990; 1998). Esterase activity was also induced in the human plasma of epileptic individuals submitted to PB treatment (Puche *et al.*, 1989).

In the present work, the effects of PB on egg eclosion, mortality, development and longevity of the mosquito *Aedes aegypti* were studied reinforcing data in literature obtained in other organisms.





## Materials and Methods

*Aedes aegypti* from São José do Rio Preto (SJRP) and Goiânia (GO) (Brazil) were used. Most of the mosquitoes from SJRP were collected in tyres and other artificial containers by the SUCEN (Superintendência de Controle de Endemias do Estado de São Paulo) and studied in the first generation produced in the Vector Laboratory, IBILCE/UNESP. The mosquitoes from Goiânia were supplied by Dr. Ionizete Garcia da Silva from the Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, GO. A difference between both populations is that GO was considered resistant to insecticides (Macoris *et al.*, 1995) and SJRP was considered susceptible (Macoris *et al.*, 1999).

The treatment with phenobarbital-PB (5-Ethyl-5phenyl-2, 4, 6 (1H, 3H, - 5H)-pyrimidinetrione) was performed by putting *A. aegypti* eggs to eclode in a PB solution where they remained till complete development. Plastic containers (12x12x5cm) with 150ml of water were used.

The experiments were tentatively conducted in three conditions, trying to find the best method for performing the work:

- Experiment 1 - 0.26mg/ml PB solution, without replacement of the liquid medium, with addition of 6mg/ml of fish food;
- Experiment 2 - 0.2mg/ml PB solution, without replacement of the liquid medium and 0.3mg/ml of food added daily to the medium;
- Experiment 3 - the same as in the experiment 2, but the liquid medium was changed in alternate days.



Experiment 3 gave the best results and then other five experiments were performed using the same protocol. The controls were prepared simultaneously under the same conditions of the tests, but without PB. Egg eclosion, mortality, development and longevity were studied. The analysis was performed daily till the death of the last adult.

Statistical analysis involved the use of Contingency  $\chi^2$  and ANOVA.



## Results

Table 1 shows the percentage of ecloded eggs and the time of reaching the different developmental stages in hours measured from the beginning of the experiment. Data on PB treatment for the mosquitoes from São José do Rio Preto (SJRP) and from Goiânia (GO) are presented. Temperature was recorded three times a day during the experiments 3 to 8 and showed a variation from 20.5°C to 27.8°C.

In relation to the egg eclosion, the difference between the treated and the control tests evaluated by Contingency  $\chi^2$  was significant in four of the eight experiments ( $P < 0.01$ ). In three of them the treated tests showed greater number of ecloded eggs values than the controls. The opposite was found in a single experiment. Difference of the total number of ecloded eggs between the treated and the control tests, analyzed by ANOVA, was not significant.

As to the time of reaching the different developmental stages, one experiment (8) showed a difference between the treated and the control tests in L1; two experiments (4 and 6), in L2; four experiments (1, 4, 6 and 8), in L3 and six experiments (1, 2, 3, 4, 6 and 8), in L4. In the pupal and adult stages, the treated tests in seven experiments (1 to 7) differed from controls. The differences between treated and control tests were greater in SJRP population.

The developmental time of each stage, for experiments 5 and 7, chosen for the visualisation of the differences between the treatments and the controls, is shown in Figures 1 and 2. A displacement to the right of the distribution picks of the treated experiment, in relation to the control, is especially evident from L3.

The mentioned difference between treated and control tests is clearly visible in the comparison of these figures.

In each experiment, adult mosquitoes were obtained in both treated and control tests, except in the treated tests of experiments 1 and 2. In the treated experiments in which adults were obtained, most of the imagoes could not emerge completely from the pupa and died in these conditions (Figure 3).

Data on the number of imagoes obtained in the control and treated tests, on the percentage of imagoes that did not emerge completely from the pupal case and on the mortality before the adult stage, in experiments 3, 4, 5 and 7, are in Table 2. The numbers of imagoes in the four experiments, including SJRP and GO, were smaller in the treated than in the control tests. Statistical analysis showed that the differences in experiments 3, 5 and 7 are significant. The frequencies of imagoes that did not emerge completely from the pupal case were approximately equal in experiments 3, 4 and 7, increasing about three times that value in experiment 5. In this experiment, the number of males incompletely emerged was more than twice the number of females. In the four experiments, mortality was high in the treated tests, mainly in experiments 3, 5 and 7.

The longevity of adults was observed in the PB treated and in the control tests (Table 3). In every case except the control from SJRP, female longevity was much greater than male longevity (almost or more than the double). In SJRP, the control female imagoes survived about 45 days and males about 41 days. In GO, the control females lived about 65 days and males about 40 days. In the treatments, the imagoes that failed to emerge, even when they were carefully taken off the pupal case with the help of a fine paintbrush, died within 24 hours.



The ones that emerged normally from the pupal case survived from 1 to 13 days.

Females also survived longer than males in both treated experiments.



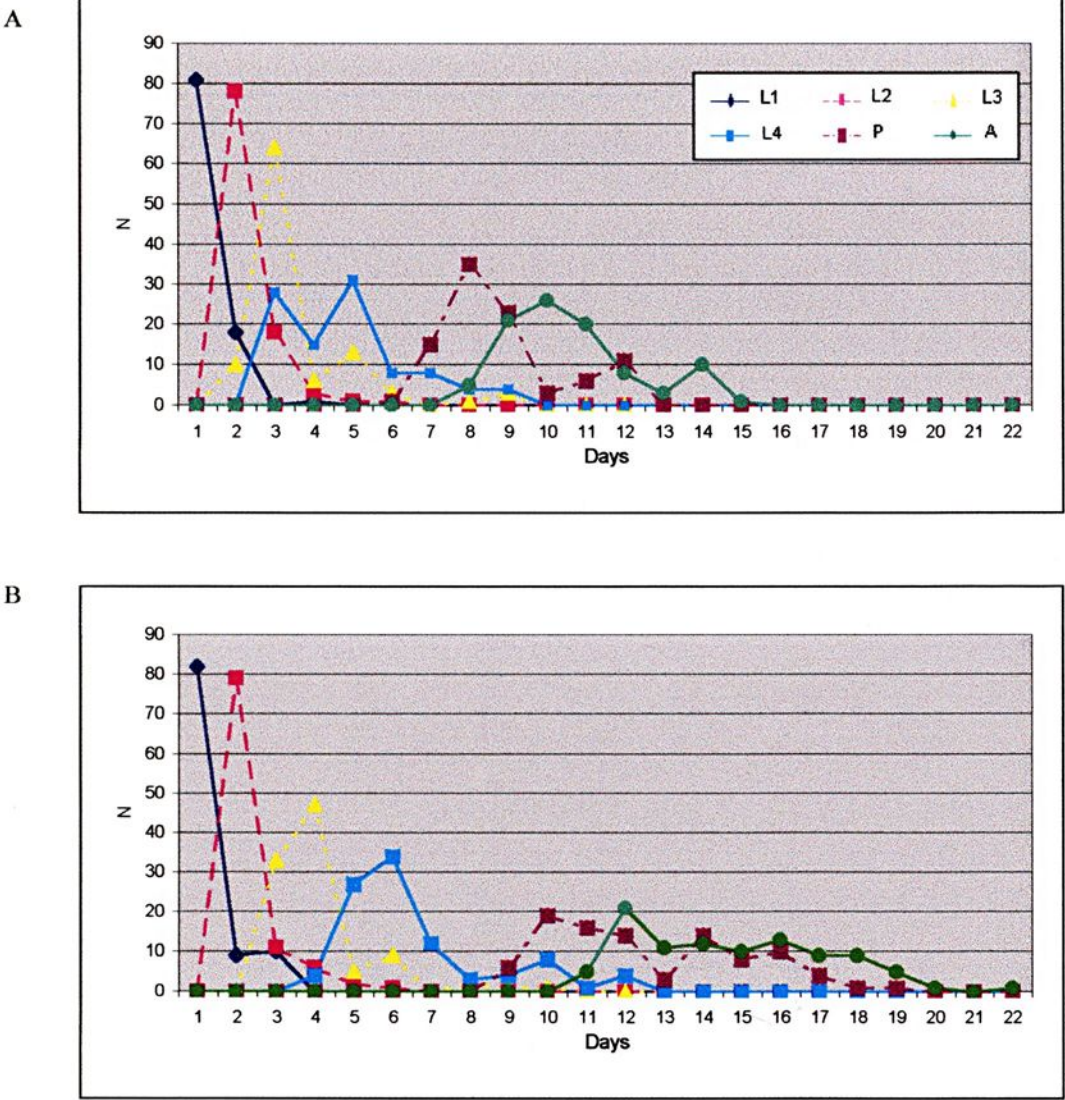


Figure 1 A-B. SJRP strain. Distribution of developmental time (in days) in each stage (L1, L2, L3, L4, P, A), in the control (A) and in the treated (B) tests of experiment 5. The symbols are the same in A and B.

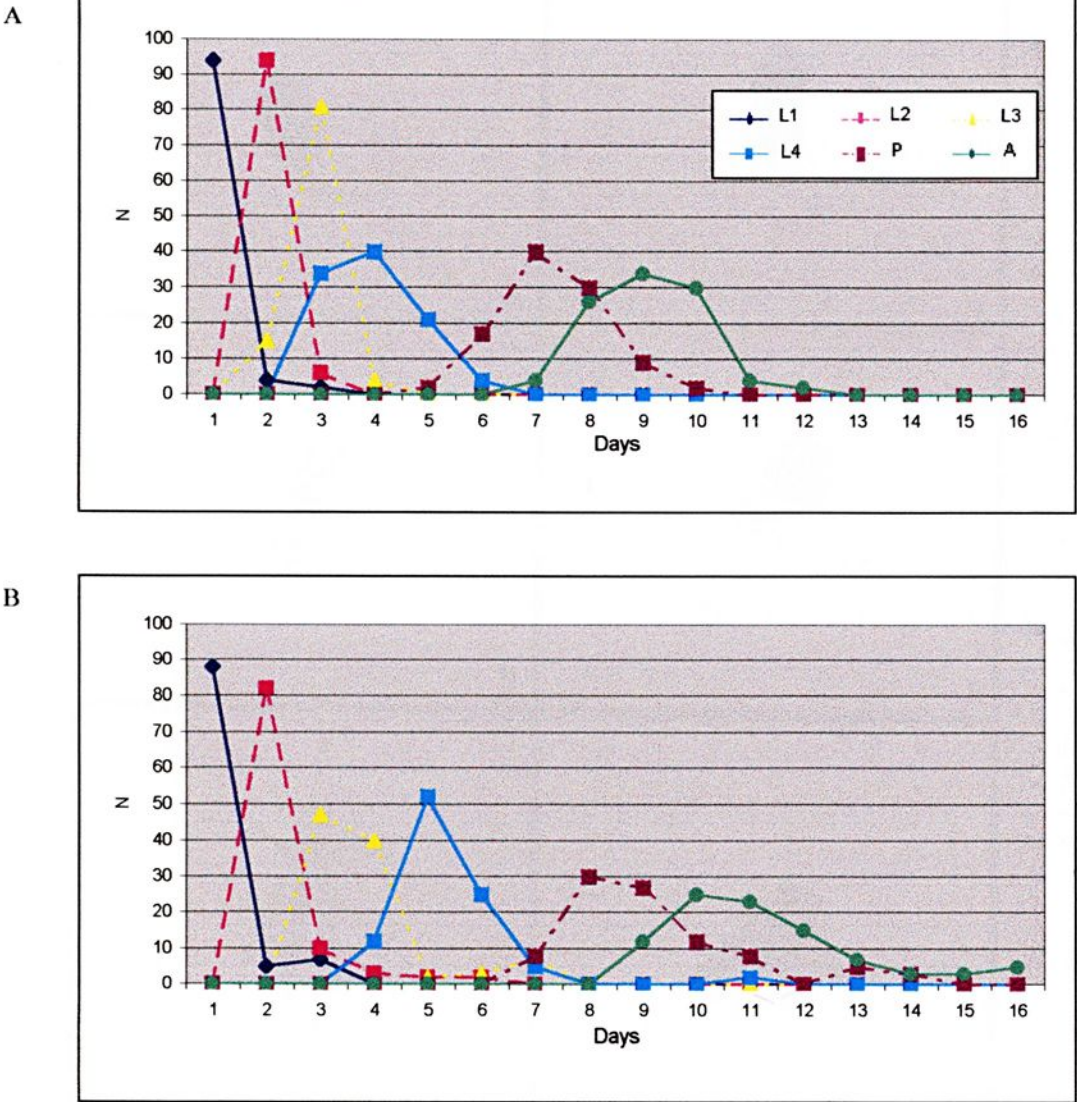


Figure 2 A-B. GO strain. Distribution of developmental time (in days) in each stage (L1, L2, L3, L4, P, A), in the control (A) and in the treated (B) tests of experiment 7. The symbols are the same in A and B.

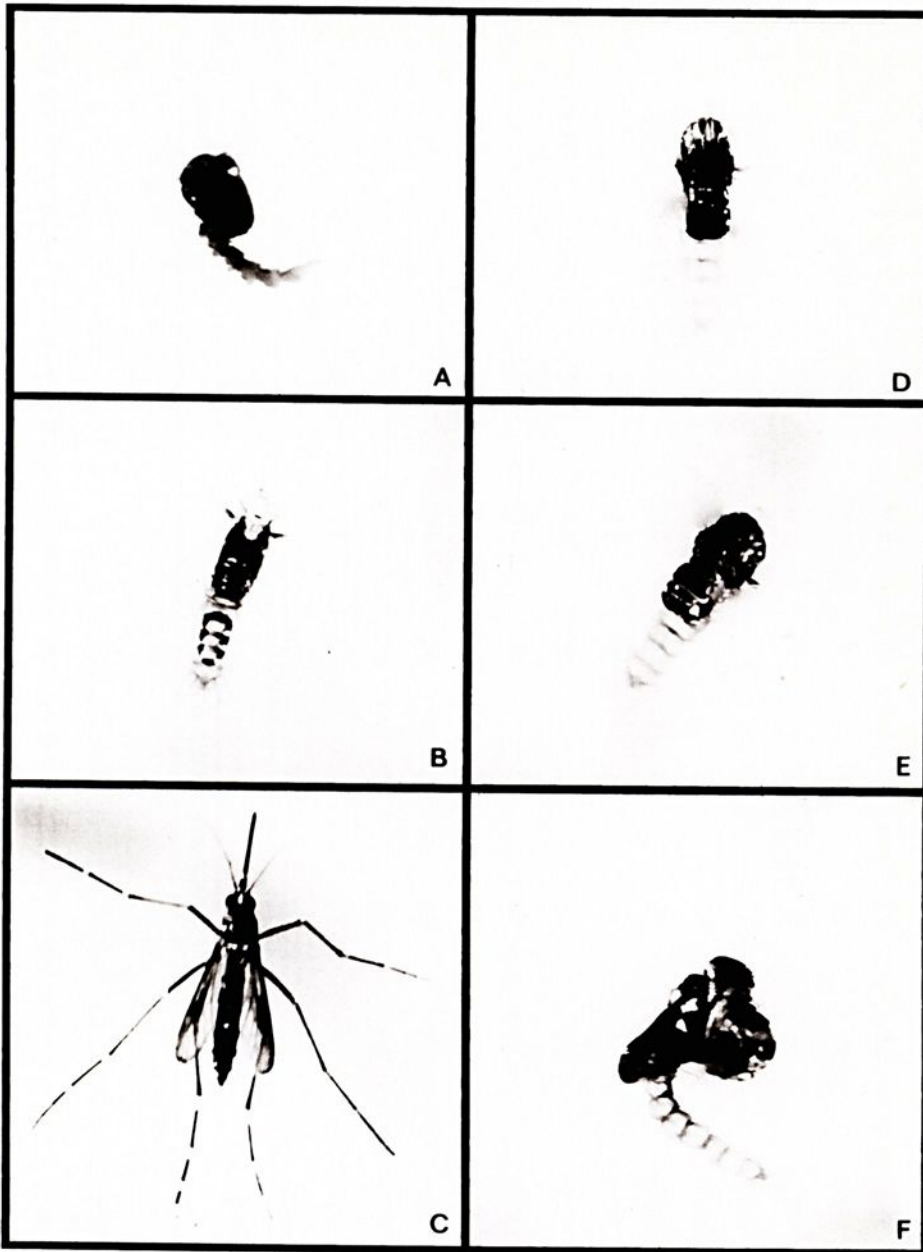


Figure 3A-F. A- Normal pupae; B- Empty pupal case; C- Adult female of *Aedes aegypti*; D-F. Three different views of a PB treated adult, partially emerged from the pupal case.



Table 1. Percentage of eclosion and time (hours) at onset of each stage, for PB treated and control mosquitoes in the experiments 1 to 6 (São José do Rio Preto) and 7 and 8 (Goiânia). L1= first instar larvae; L2= second instar larvae; L3= third instar larvae; L4= fourth instar larvae; P= pupae; A= adult; C= control; T= treated. \*P<0.01

| Origin | Exp | Test | N.eggs | Eclosion (%) | Time at onset of each stage (hours) |    |     |     |     |     | Temperature |      |      |
|--------|-----|------|--------|--------------|-------------------------------------|----|-----|-----|-----|-----|-------------|------|------|
|        |     |      |        |              | egg-L1                              | L2 | L3  | L4  | P   | A   | Min.        | Max. | Mean |
| SJRP   | 1   | T    | 141    | 92.9         | 24                                  | 48 | 96  | 168 |     |     |             |      |      |
|        |     | C    | 118    | 91.5         | 24                                  | 48 | 72  | 120 | 168 | 264 |             |      |      |
|        | 2   | T    | 150    | 69.3         | 24                                  | 48 | 72  | 120 | 264 |     |             |      |      |
|        |     | C    | 110    | 70.9         | 24                                  | 48 | 72  | 96  | 168 | 288 |             |      |      |
|        | 3   | T    | 185    | 63.2         | 24                                  | 48 | 72  | 120 | 192 | 264 | 22.0        | 26.7 | 24.8 |
|        |     | C    | 146    | 56.0         | 24                                  | 48 | 72  | 96  | 144 | 192 |             |      |      |
|        | 4   | T    | 100    | 44.0*        | 24                                  | 72 | 96  | 144 | 192 | 240 | 25.0        | 28.0 | 26.6 |
|        |     | C    | 100    | 26.0         | 24                                  | 48 | 72  | 96  | 144 | 192 |             |      |      |
|        | 5   | T    | 100    | 94.0*        | 24                                  | 48 | 72  | 96  | 216 | 264 | 26.0        | 29.0 | 27.7 |
|        |     | C    | 100    | 80.0         | 24                                  | 48 | 72  | 96  | 168 | 216 |             |      |      |
|        | 6   | T    | 100    | 76.0*        | 24                                  | 96 | 144 | 216 | 264 | 336 | 16.0        | 25.0 | 20.5 |
|        |     | C    | 100    | 99.0         | 24                                  | 48 | 96  | 144 | 192 | 264 |             |      |      |
| GO     | 7   | T    | 100    | 60.0         | 24                                  | 48 | 72  | 96  | 168 | 216 | 26.0        | 29.0 | 27.8 |
|        |     | C    | 100    | 47.0         | 24                                  | 48 | 72  | 96  | 144 | 192 |             |      |      |
|        | 8   | T    | 100    | 94.0*        | 48                                  | 72 | 120 | 144 | 240 | 312 | 16.0        | 25.0 | 20.5 |
|        |     | C    | 100    | 82.0         | 24                                  | 72 | 96  | 120 | 240 | 312 |             |      |      |



Table 2. Imago production (percentage) in the control (C) and treated (T) tests and percentage of partially emerged imago (PEI). Mo= mortality from larvae to the emergence from pupal case; F=female; M=male; T=total; NL1= number of first instar larvae in the experiments. \*  $P < 0.01$

| Origin | Exp | Test | NL1 | Imago production |       |       | PEI   |       |       |       |
|--------|-----|------|-----|------------------|-------|-------|-------|-------|-------|-------|
|        |     |      |     | Total            | F     | M     | Total | F     | M     | Mo    |
| SJRP   | 3   | T    | 87  | 27.6*            | 11.5  | 16.1  | 18.4  | 10.3  | 8.1   | 72.4* |
|        |     | C    | 37  | 89.2             | 21.6* | 67.6* |       |       |       | 10.8  |
|        | 4   | T    | 44  | 68.2             | 36.4  | 31.8  | 20.4  | 13.6  | 6.8   | 31.8  |
|        |     | C    | 26  | 88.5             | 50.0  | 38.5  |       |       |       | 11.5  |
|        | 5   | T    | 94  | 33.0*            | 20.2  | 12.8  | 62.8  | 18.1* | 44.7* | 67.0* |
|        |     | C    | 80  | 93.7             | 41.2  | 52.5  |       |       |       | 6.3   |
| GO     | 7   | T    | 60  | 73.3*            | 36.7  | 36.7  | 20.0  | 8.3   | 11.7  | 26.7* |
|        |     | C    | 47  | 100              | 51.1  | 48.9  |       |       |       | 0     |

Table 3. Adult longevity in days. T= PB treated; C= control; N= number of females (F) and males (M).

| Origin | Test | Adult longevity |    |         |    |         |    |      |      |
|--------|------|-----------------|----|---------|----|---------|----|------|------|
|        |      | N               |    | Minimum |    | Maximum |    | Mean |      |
|        |      | F               | M  | F       | M  | F       | M  | F    | M    |
| SJRP   | T    | 14              | 24 | 1       | 1  | 8       | 4  | 2.5  | 1.3  |
|        | C    | 21              | 41 | 8       | 9  | 53      | 51 | 44.6 | 41.5 |
| GO     | T    | 17              | 12 | 1       | 1  | 13      | 3  | 4.1  | 1.2  |
|        | C    | 10              | 11 | 47      | 26 | 77      | 58 | 65.7 | 39.3 |

## Discussion

Data in literature suggest that the organisms respond to PB treatment by the induction of the expression of one or several genes. Brun *et al.* (1996) showed that the expression of cytochrome P450 gene in *Drosophila melanogaster* is under the control of that substance, with induction factor of 15-fold in some tissues. Cytochrome P450 metabolizes a variety of endogenous and exogenous chemicals including aromatic compounds, steroids, fatty acids, etc., and is also involved in insecticide resistance. The induction mechanism by PB seems to be complex, involving removal of the repressor that binds on a specific sequence 5' of a cytochrome P450 gene (Brun *et al.*, 1996).

In the present, study the biological effects of PB treatment, including percentage of egg eclosion, developmental time, mortality from larval stage till the emergence from pupal case and adult longevity were analysed in two strains of *Aedes aegypti*, being one of them considered insecticide susceptible (SJRP) and the other considered insecticide resistant (GO). The effects are indicative that important changes occurred in response to the PB action during insect development, in both cases.

The percentage of eclosion apparently was not affected by PB action. But PB treatment produced a delay in the developmental time that increased as the stages progressed. Although some variation had occurred among the eight experiments, the number of them that showed differences between treated and control tests as to the developmental time increased from one in L1 (experiment 8) to six in L4 (experiments 1 to 4, 6 and 8) and seven in pupae and adults





(experiments 1 to 7). Differences of four days were detected at the pupal stage and of three days at both L4 and adult stages. As expected, the lowest temperatures that occurred during experiments 6 and 8 increased the developmental time in both treated and control tests.

Although delay in development has occurred in *A. aegypti* from São José do Rio Preto and from Goiânia treated with PB, the differences between control and treated tests were greater in SJRP than in GO. Besides, the female longevity was greater in GO than in SJRP. As mentioned before, GO was considered resistant to OP insecticides while the SJRP population was considered susceptible to the same compounds. Then, the differences on PB effects might be due to the greater resistance level of GO population. Fuchs *et al.* (1994) observed that resistant and susceptible *Drosophila* respond differently to PB treatment, the first showing a lower inducibility of the cytochrome P450 system than the second. According to the mentioned authors this is due to a greater PB degradation in resistant insects.

In the first experiment in which a higher PB dose was used (0.26mg/ml instead of 0.2mg/ml used in the seven other experiments), pupae were not obtained, that is, the development was blocked at the larval stages. All the other experiments, except one (experiment 2) yielded adults, although in number smaller than in controls.

The effects of PB were also observed at the pupal stage, acting by blocking the imago emergence from pupal case. A relatively high percentage of imagoes died during emergence, with the body partially out of the pupal case. Even if they were carefully helped to get out from the pupal case, they died in a short period of



time (about 24 hours or less). This observation suggests that the impossibility to emerge is not the cause of their death but an indication that a deeper problem is involved. The slow movements of the adults from the treated experiments suggest that this problem may involve the nervous system control. This suggestion is supported by literature. As mentioned, several studies indicated an effect of PB on the nervous system development. Loegering and Johnson (1994) showed that, in rat, PB at 30 $\mu$ g/ml reduced the percentage of neurons with dendrites from 20% to 50% of the controls. Also the number of primary dendrites per neuron and the average length per dendrite were significantly reduced. Johnson *et al.* (1996) also showed that, in rat cultures, dendritic development and maintenance of dendritic morphology are affected by PB at 15 $\mu$ g/ml. However, with the use of PB at 30  $\mu$ g/ml Johnson *et al.* (1998) demonstrated that the deleterious effects of PB can be reversed if the exposure to PB is in a short period of time. In another study, PB at 50 to 75mg/kg intraperitoneal triggered widespread apoptotic cell death in the infant rat brain (Ikonomidou *et al.*, 2000). Besides, some authors have reported brain acetylcholinesterase (AChE) inhibition following pretreatment with monooxygenase inducers such as PB (Chambers and Chambers, 1990). It has been shown that PB can decrease the release, alter the rate of synthesis and change the concentration of acetylcholine (Sudha *et al.*, 1996). Changes in mitochondrial enzymes, cytochrome oxidase and NADH-cytochrome-c-redutase were also detected in whole brain homogenates (Benzi *et al.*, 1979).

Other data in literature showed that agents that potentiate the action of  $\gamma$ -aminobutyric acid (GABA) (such as PB), as well as those that decreases brain levels of GABA (such as allylglycine) affects brain development. When the

convulsant allylglycine was fed to larvae of *D. melanogaster*, they pupated and matured apparently normally, but viable adults failed to emerge. According to the authors, allylglycine is likely to affect some process that is important for adult viability, but is missing or is not essential in larvae (Howard *et al.*, 1975). The same might be happening with the effect of phenobarbital, in this work.

In the present study using PB in a continuous treatment of *A. aegypti*, the development time delay, the impossibility to emerge from pupal case and the shorter longevity of the adults indicate that important biological processes are being affected in the treated mosquitoes.

Because PB is used as an anticonvulsant in both pregnant mothers and neonates with seizures, there has been an increased concern over its potential adverse effects on the development of the nervous system (Loegering and Johnson, 1994; Ikonomidou *et al.*, 2000). We may consider the present results as reinforcing the idea that there is a risk in the consumption of PB that deserves attention.

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## V. ARTIGO 3

### **EFFECT OF PHENOBARBITAL ON INSECTICIDE RESISTANCE AND ON THE ESTERASE PATTERN OF *Aedes aegypti***

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temephos

## Resumo

O envolvimento do PB no aumento de tolerância ao temephos (organofosforado), em larvas de 4º estágio (L4) de *Aedes aegypti*, foi analisado nos tratamentos contínuo (de ovo a L4) e descontínuo (30 horas). Foram utilizadas amostras de São José do Rio Preto (SJRP) e Goiânia (GO – considerada resistente a inseticidas). Observou-se aumento significativo na tolerância ao temephos em larvas pré-tratadas com PB, nos dois tratamentos, na concentração de 0,01mg/L de temephos, mas o tratamento contínuo mostrou níveis mais altos em 0,02mg/L de temephos. As taxas de redução da mortalidade foram maiores em SJRP do que GO. A análise dos efeitos do PB, no padrão de esterases, mostrou que o *locus* contendo genes para EST-14 (colinesterase) e para EST-13 (possivelmente um alelo do mesmo *locus*), pode estar envolvido na resistência induzida pelo PB. O butóxido de piperonila (PBO), inibidor de oxidases, utilizado em larvas pré-tratadas com PB indicou que as oxidases não estão envolvidas na indução da resistência por essa substância. Quando larvas pré-tratadas com PB foram submetidas ao PBO e em seguida ao temephos, observaram-se níveis mais altos de tolerância, sugerindo que PB e PBO estão atuando em sinergismo. O não envolvimento das oxidases reforça a indicação do papel das esterases na resistência induzida pelo PB.



## Abstract

The involvement of phenobarbital (PB) in the increase of tolerance to temephos (organophosphorous) in fourth instar larvae of *Aedes aegypti* was analyzed in continuous (from egg to fourth instar larvae) and discontinuous (30 hours) treatments. Mosquitoes from São José do Rio Preto (SJRP) and Goiânia (GO - considered resistant to insecticides) were used. Significant increased tolerance in PB pretreated larvae occurred in both types of treatments at 0.01mg/l temephos concentration, but only the continuous treatment showed significant increased tolerance at 0.02mg/l temephos. The mortality reduction rates were greater for SJRP than for GO. Esterase patterns were examined in PB pretreated larvae showing that the locus containing genes for esterase band EST-14 (a cholinesterase) and for band EST-13 (supposed to be an allele of the same locus) is involved in the induction. Piperonyl butoxide (PBO) used to detect function of oxidases in the PB induced resistance suggested that the oxidases are rather not involved. When PB pretreated mosquitoes were submitted to PBO followed by temephos, increased tolerance was observed suggesting that PB and PBO are acting in synergism. The indication of uninvolved of oxidases in the PB induced tolerance in *Aedes* reinforces the role of esterases in the insecticide resistance.

## Introduction

The induction is a process in which a chemical stimulus enhances the activity of the detoxification system by the production of additional enzymes (Terriere, 1984). A wide variety of chemicals have been shown to elicit the inductive response; among them are included insecticides and the phenobarbital (PB). Studies of detoxification in insects are important because on the one hand resistance is involved with the control of vectors and pests and on the other hand it is an important aspect in the adaptation of insects to the environment.

Organophosphate (OP), substances widely used as insecticides, are known to be detoxified by several enzymes in the mammals and insects, such as carboxylesterases, oxidases and glutation-S-transferases (GSTs). These enzymes act as a detoxifying mechanism by reducing the amount of free OP molecules (Kaliste-Korhonen, Tuovinen & Hänninen, 1998).

PB have been used in the research of OP insecticide toxicity in flies (Scott & Lee, 1993; Fuchs, Spiegelman & Belitsky, 1994) and rodents (Clement, 1983; Kaliste-Korhonen et al., 1990). It has been shown to decrease moderately the toxicity of some OPs, as measured by the increase of LD<sub>50</sub> values. For the OP soman, this increase was by 1 to 12-fold for rats and by 1 to 3-fold for mice (Dubois & Knoshita, 1968; Clement, 1983). Such changes have been thought to result from induction of carboxylesterases, cholinesterases, hepatic paraoxonase or liver cytochrome P450 enzymes (Chambers et al., 1994; Kaliste-Korhonen, Tuovinen & Hänninen, 1998). In rat liver the activity of three distinct

carboxylesterases was significantly induced (3 to 8-fold) by the administration of phenobarbital (Ali et al., 1985).

As shown by Kaliste-Korhonen, Tuovinen and Hänninen (1998), in rodents, in contrast to the induced esterases, serum paraoxonase activity was decreased by PB treatment, but acetylcholinesterase activity in erythrocytes and cholinesterase activity in the diaphragm was slightly increased. Carboxylesterase activity was also elevated in the liver cytosol. Cytochrome P450 enzymes, although greatly induced, did not reduce the toxicity of paraoxon. It was also shown that the OP detoxification capacity of cytochrome P450 enzymes may vary with different OPs.

According with Terriere (1984), the detoxification systems in insects are identical with those of higher animals and many of the chemicals that induce enzymes in mammals are also active in insects. In insects, there is evidence of the induction of the three most important systems of insecticide detoxification that are the microsomal oxidases, glutathione S-transferases and the carboxylesterases (Terriere, 1984). As mentioned, cholinesterases were also induced in some resistance studies.

In *Drosophila melanogaster* and *Musca domestica*, PB induced tolerance related to cytochrome P450 in insecticide susceptible strains compared to insecticide resistant ones (Scott & Lee, 1993; Fuchs, Spiegelman & Belitsky, 1994).

Scott and Lee (1993) suggested a possible link between regulation of insecticide resistance and PB induction. Plapp (1984) believes that the two phenomena may involve the same genetic mechanism and that induction can



result in this type of resistance. According to this view, it involves the regulator genes or their product - a cytoplasmic receptor. A more sensitive receptor, responding earlier to insecticides in the insect's environment, would reduce toxicity by allowing more rapid metabolism of the toxicant. However, the mechanism (s) by which PB increases expression of P450 in eukaryotes remains unknown. A previous hypothesis of Terriere (1984) that the same regulatory genes are responsible for both induction and monooxygenase-mediated insecticide resistance is not refuted (Liu & Scott, 1997).

Relation between esterase activity and resistance to OP is well documented for a large number of insects. Resistant mosquitoes from many places in the world have been characterized over the last years, revealing amplification of several closely related esterases. In the mosquito *Culex pipiens* and peach-potato aphid *Myzus persicae*, insecticide resistance is due to overproduction of carboxylesterases resulting from gene amplification that detoxify the insecticide by sequestration (Devonshire, 1977; Fournier et al., 1987; Cuany et al., 1993; Callaghan et al., 1998). *Aedes aegypti* mosquitoes already presented resistance to insecticides related with elevated esterase activity (Mourya, Hemingway & Leake, 1993; Mazzarri & Georghiou, 1995; Vaughan, Chadee & Ffrench-Constant, 1998).

In Brazil, insecticides, including the organophosphate temephos, have been extensively used for routine control of *A. aegypti* (Lima-Catelani, 1996). Our previous work showed the presence of multiple changes in the esterase band patterns in the resistant population from Goiânia compared to the susceptible population from São José do Rio Preto (SJRP) (both from Brazil), indicating the



involvement of some esterases with resistance in this vector (Sousa-Polezzi & Bicudo a, to be published elsewhere). These results and the mentioned data in literature suggested the present study on PB effects. We intended to know if PB interferes in the resistance degree of *A. aegypti*, the important vector of dengue, hemorrhagic dengue and yellow fever, as observed in other organisms and, in positive case, if the PB causes changes in esterase patterns reinforcing the role of these enzymes on the increased insecticide resistance of Brazilian populations from SJRP and GO.



## Materials and methods

### *Mosquitoes*

*Aedes aegypti* mosquitoes from São José do Rio Preto-SP (SJRP) and Goiânia-GO (Brazil) were analyzed. In SJRP they were collected by the SUCEN (Superintendência de Controle de Endemias do Estado de São Paulo) and those from Goiânia were supplied by Dr. Ionizete Garcia da Silva from the Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, Goiânia-GO.

### *Phenobarbital Treatment*

Phenobarbital (PB) (Gardenal®, Rhodia Farma, Brasil) treatments were used for studying insecticide resistance changes and for detecting esterase pattern alterations. In the first case, two kinds of treatments with PB were performed: (1) continuous treatment in which *A. aegypti* eggs were put to eclode in 0.2mg/ml PB water solution in which they remained till fourth instar larvae (L4) and (2) discontinuous treatment in which L4 were treated during 30 hours in medium containing 0.26mg/ml of PB. Simultaneously, the controls were submitted to the same conditions, but without PB. The PB pretreated L4 and the controls were submitted to temephos bioassay.

In the study of esterase pattern, the PB treatments were the same but, in the continuous treatment, the mosquito developed till adult stage and, in the discontinuous treatment, besides larvae, also pupae and adults were treated during 30 hours each. L4, pupae and adults were frozen and analyzed as to the esterase band patterns in polyacrylamide gels with a period of one month.

### *Temephos Bioassay*

For evaluation of susceptibility to temephos, PB pretreated and not treated L4 from SJRP and GO were submitted to that organophosphate (Abate®, Cyanamid, Brasil), at three concentrations: 0.005mg/l, 0.01mg/l and 0.02mg/l. In each test, groups of 10 larvae were exposed to each insecticide concentration in a Petri dish holding 40ml of medium. For control, simultaneously to the tests with temephos, groups of 10 larvae were put in medium with only PB in the used dose or only water. The mortality was accessed 24 hours after larval exposure. After the tests, larvae were frozen to analyze esterase pattern in polyacrylamide gels.

### *Piperonyl Butoxide Bioassay*

The action of oxidase inhibitor piperonyl butoxide (PBO) (Acros Organics, U.S.A.) was investigated by exposing PB pretreated (discontinuous) and not treated L4 to a 0.006µl/ml PBO concentration during 24 hours in the presence of temephos insecticide at 0.01mg/l, using groups of 10 larvae. Tests involving exposition of PB treated larvae first to the PBO and after to the temephos were also done, using PBO at 0.01µl/ml concentration during 4 hours. In both PBO concentration (0.006µl/ml and 0.01µl/ml) no mortality occurred. The mortality was accessed 24 hours after larval insecticide exposure.

### *Esterase Analysis*

L4, pupae and male and female adults from continuous and discontinuous PB treatments, and treated and controls fourth instar larvae from temephos bioassay were individually used for sample preparations. In this study, 8% polyacrylamide gels (PAGE) were used (Laemmli, 1970). The mosquitoes were

individually squashed in 25  $\mu$ l of the buffer solution (1.5M Tris-HCl, pH8.8, plus 10% glycerol) for each sample preparation. The supernatant (10 $\mu$ l) was then used for each sample. Electrophoresis was performed for 2 hours at  $\sim$ 25°C using a constant 200V. The running buffer used was 0.1M Tris-glycine at pH 8.3. Esterases were identified in the gels as described by Johnson et al. (1966) and Steiner and Johnson (1973), including preincubation (45min.) at room temperature ( $\sim$ 25°C) in 50 ml 0.1M sodium phosphate at pH 6.2, and staining reaction in the dark (1 hr.) with a solution containing both  $\alpha$ - and  $\beta$ -naphthyl acetate as substrates (30mg of  $\alpha$ - and 15mg of  $\beta$ -naphthyl acetate), 60mg of fast blue and 5ml of N-propanol in 50ml of the sodium phosphate solution. The gels were dried at room temperature using gelatin and cellophane wound slab gels in an embroidering hoop, as described by Ceron, Santos and Bicudo (1992). The esterase bands in gels were numbered following Lima-Catelani (1996), from the less anodic to the more anodic end of the gels.

#### *Esterase Band Activity*

The Global Lab Image Program (Data Translation) was used for evaluating the activity level of esterase bands in polyacrylamide gels, indicated by thickness and staining intensity. In this program, the full range of grayscale values goes from 0 (complete black) to 255 (complete white). The values attributed by the program for each band were used for statistical analysis.





*Statistics*

Significance of the difference between mean values of tests and controls in all the experiments involving comparisons was assessed by homogeneity test (Contingency  $\chi^2$ ).



## Results

### *Susceptibility in temephos bioassay of mosquitoes pretreated with phenobarbital (PB)*

Table 1 shows the results of susceptibility to the temephos obtained after the pretreatments with PB accomplished in discontinuous and continuous ways and in the controls, for both populations (SJRP and GO).

The experiments included three types of control tests using fourth instar larvae, being one prepared with the larvae put into water, another put into medium with PB and the third one put into temephos medium in three different concentrations. In the two first types of controls, the larva survival was 100%. The mortality in the third control test followed the temephos concentration being greater as that concentration increased (dose dependent) for both populations, in the continuous as well as in the discontinuous treatments.

In the susceptibility tests involving the PB pretreatment, the mortality was also greater as the temephos concentration increased.

Contingency  $\chi^2$  was used in the comparison of mortality ratio between pretreated and not treated PB tests, in both populations, in discontinuous and continuous treatments (Table 2). In every case, mortality decrease was observed in the PB pretreated mosquitoes but only at 0.01mg/l temephos concentration such decrease was significant in every comparison of both tests. The decreases were also significant at 0.005mg/l in the discontinuous pretreatment of SJRP and in both populations, at 0.02mg/l concentration, in the continuous pretreatment.

Comparison between discontinuous and continuous treatments showed mortality ratio higher in the first than in the second tests, in every comparison of

GO mosquitoes, while in SJRP the discontinuous tests showed higher values in two comparisons (PB→TE, 0.01mg/l and 0.02mg/l) and lower values in one comparison (PB→TE, 0.01mg/l). However, the statistical analysis showed that the differences in all of these comparisons were not significant (Table 3).

The comparison between both populations as to the mortality ratio of mosquitoes PB pretreated and not pretreated, exposed to temephos, showed higher values for SJRP in both experiments. Contingency  $\chi^2$  showed significant differences for every comparison, except at 0.01mg/l temephos concentration in the PB pretreatment discontinuous test (Table 4).

The Figure 1 shows the variations of temephos susceptibility among the three concentrations used. The graphs summarize all data on mortality obtained in the experiments.

*Effect of the phenobarbital (PB) on the esterase patterns: changes in band frequencies*

Polyacrylamide gels from individuals submitted to continuous and discontinuous PB treatments compared to the control (water medium) showed some changes in the esterase patterns of PB treated mosquitoes. The changes induced by PB involved increase or decrease in the frequency of some bands (Table 5).

In the discontinuous treatment, significant differences in the frequency of esterase bands compared with the control were observed only in larvae, involving frequency decrease of bands EST-2 and EST-20 (Table 6).

The continuous PB treatment caused significant frequency reduction of the bands EST-2, EST-4 and EST-20 (Table 6). A single band, EST-14, showed significant increase in larvae ( $P < 0.01$ ) and pupae ( $P < 0.05$ ).

*Effect of continuous treatment with phenobarbital (PB) on the activity degree of esterases*

Bands EST-1, EST-13 and EST-14 showed the greatest alterations of staining degree and thickness in the continuous treatments with PB. In the discontinuous tests these changes were not so evident.

Such variations were quantified in pupae by image analysis and statistically analyzed by Contingency  $\chi^2$ . Pupae were chosen because they showed the greatest activity increase of the bands EST-13 and EST-14 and activity decrease of the EST-1. Larvae also showed the activity increase of bands EST-13 and EST-14 but in lower degree than in the pupae. Band EST-1, in larvae, showed the same degree of activity decrease that was observed in pupae. The PB treated adults also showed activity decrease of EST-1 mainly in imagoes that not emerged completely from the pupal case (Sousa-Polezzi & Bicudo b, to be published elsewhere).

Means and standard deviation obtained for each band and the significance of the differences between treated and controls are in Table 7. Figure 2 contains the representative graphs of the activity degree of these esterase bands. The statistical tests showed significant differences between treated pupae and controls for esterases EST-1 and EST-13.



*Effect of both phenobarbital (PB) and temephos (TE) treatments on the esterase patterns*

Polyacrylamide gels were also used to analyze the esterase changes in larvae PB pretreated or not treated, exposed to temephos bioassay. The changes caused by administration of temephos alone involved the inhibition of several esterases in the mosquitoes of both localities. EST-3 to EST-6 and EST-18 were completely inhibited and EST-A to EST-D, EST-7, EST-12 to EST-14 and EST-20 were partially inhibited. Only EST-1 was completely preserved. PB pretreatment followed by exposition to temephos maintained the same results (Figure 3).

*Effect of piperonyl butoxide (PBO) on mortality of L4 mosquitoes exposed to temephos*

Mortality decrease occurred in both experiments involving PBO exposition of mosquitoes PB pretreated, followed by temephos tests, and in tests in which PBO exposition was simultaneous with temephos. However, in the first case the mortality decrease was lower than in the second type of experiment. In both cases the mortality was significantly lower than in those experiments in which the mosquitoes were not exposed to PBO (Table 8).

In the experiments that were not PB pretreated, the mortality differences between the experiments with exposure to temephos alone and the experiments PBO pretreated followed by temephos exposure were not significant (Table 8).

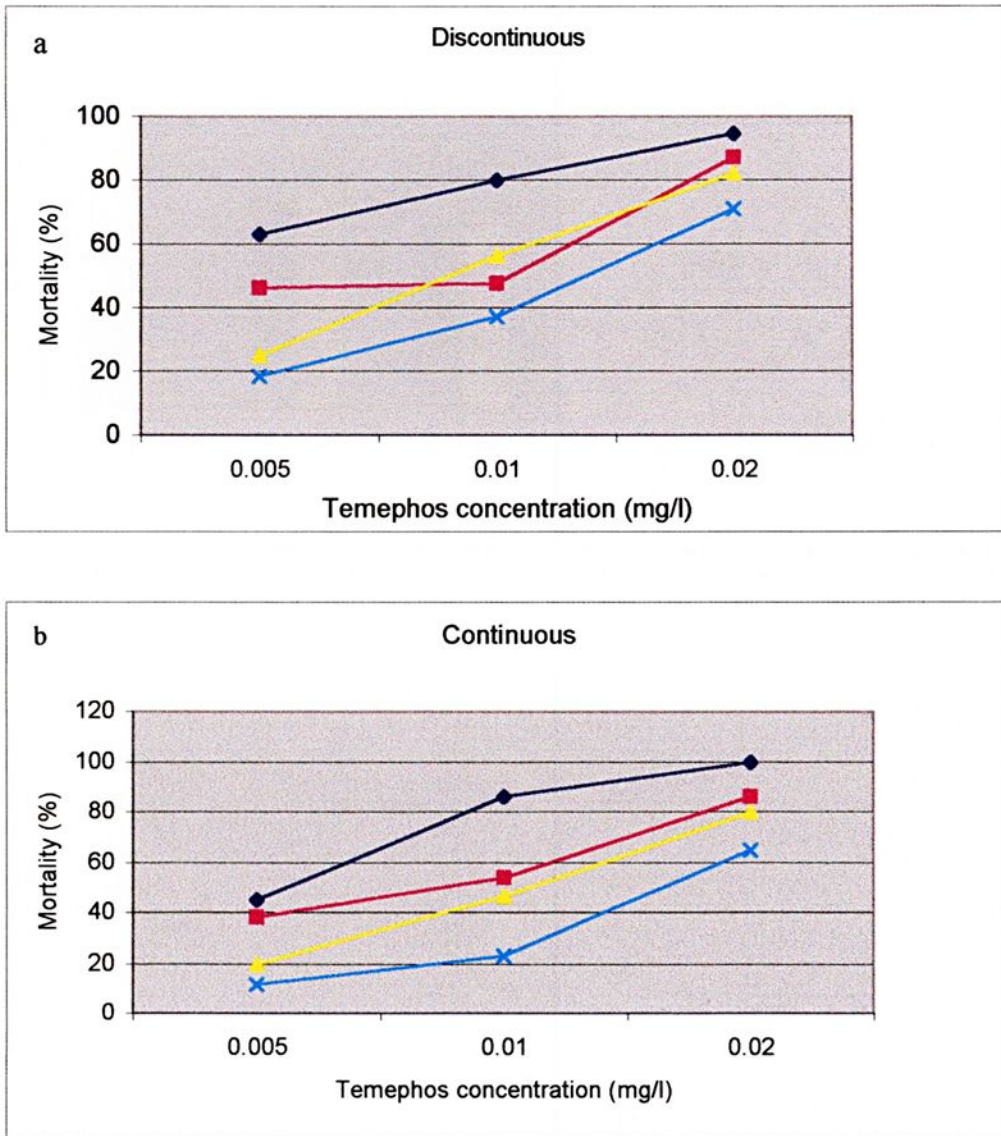
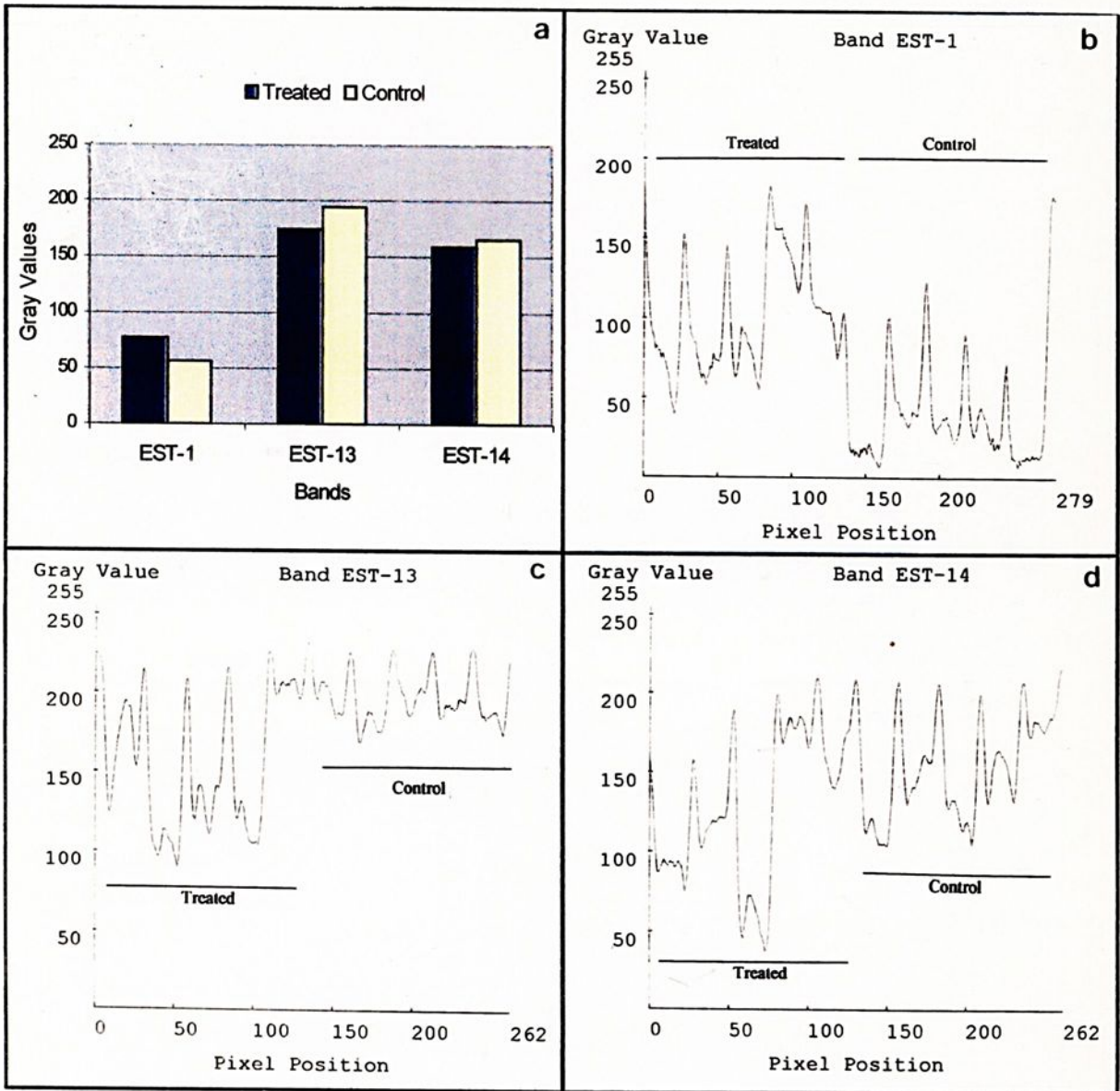


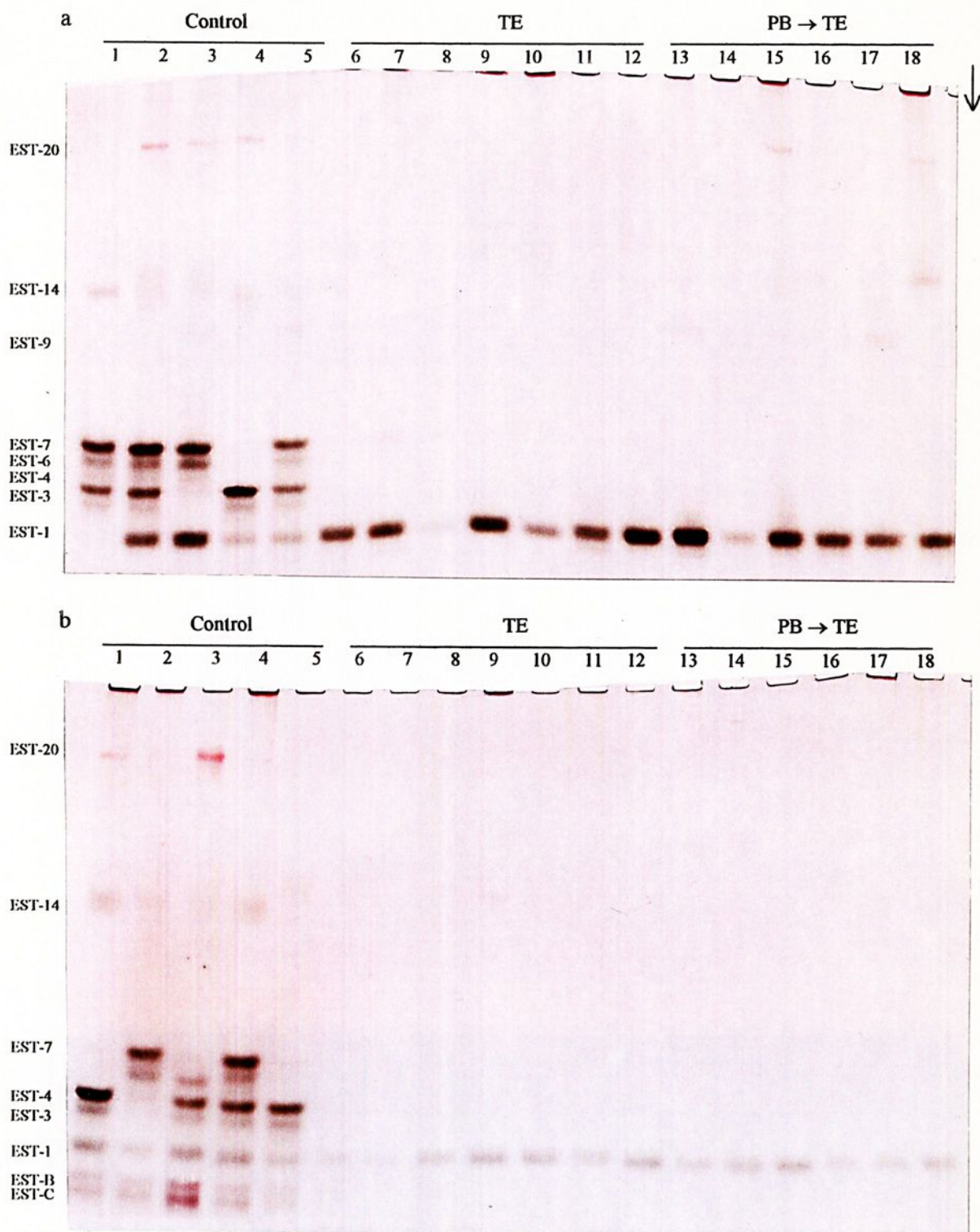
Figure 1. Graphs for temephos susceptibility tests of mosquitoes from SJRP and GO, submitted (PB → TE) or not (TE) to discontinuous (a) and continuous (b) PB pretreatments. PB= phenobarbital; TE= temephos.

—◆— SJRP - TE; —■— SJRP-PB→TE; —▲— GO - TE; —×— GO-PB→TE.



**Figure 2.** (a) Graph for activity degree mean values in pixels of esterase bands EST-1, EST-13 and EST-14 in continuous PB (phenobarbital) pretreated and control mosquitoes from SJRP. (b-d) Profile graphs of the same bands, obtained in Global Lab Image program for illustrating the way the data in (a) were obtained.





**Figure 3.** Esterase patterns in polyacrylamide gel of larvae submitted to PB and temephos treatment. (a) São José do Rio Preto (SJRP); (b) Goiânia (GO).



*Table 1.* Temephos susceptibility tests for control (A= without PB pretreatment, larvae put into water; PB= PB pretreated mosquitoes put into water; TE= without PB pretreatment, put into medium with three different temephos concentrations-mg/l) and for discontinuous and continuous PB pretreated mosquitoes put into medium with the same three temephos concentrations (PB→TE). N= number of larvae used; M= mortality percentage. Observations done 24 hours after preparation of tests.

| Treatment     | Population | Control |   |     |   |       |      |      |      |      |       | Tests   |      |      |      |      |      |
|---------------|------------|---------|---|-----|---|-------|------|------|------|------|-------|---------|------|------|------|------|------|
|               |            | A       |   | PB  |   | TE    |      |      |      |      |       | (PB→TE) |      |      |      |      |      |
|               |            |         |   |     |   | 0.005 |      | 0.01 |      | 0.02 |       | 0.005   |      | 0.01 |      | 0.02 |      |
|               |            | N       | M | N   | M | N     | M    | N    | M    | N    | M     | N       | M    | N    | M    | N    | M    |
| Discontinuous | SJRP       | 150     | 0 | 150 | 0 | 65    | 63.0 | 40   | 80.0 | 55   | 94.5  | 65      | 46.1 | 40   | 47.5 | 55   | 87.2 |
|               | GO         | 320     | 0 | 320 | 0 | 135   | 25.2 | 110  | 56.4 | 90   | 82.2  | 135     | 18.5 | 110  | 37.3 | 90   | 71.1 |
| Continuous    | SJRP       | 50      | 0 | 50  | 0 | 60    | 45.0 | 50   | 86.0 | 60   | 100.0 | 60      | 38.3 | 50   | 54.0 | 50   | 86.0 |
|               | GO         | 50      | 0 | 50  | 0 | 70    | 20.0 | 60   | 46.6 | 70   | 81.4  | 50      | 12.0 | 60   | 23.3 | 60   | 65.0 |

*Table 2.* Mortality decrease in percentage and its significance in the comparison between PB pretreated and not treated experiments in exposition to temephos, in SJRP and GO populations. \*=  $P < 0.05$ ; \*\*=  $P < 0.01$

| Comparisons | Treatment     | Population | Temephos concentration (mg/l) |        |        |
|-------------|---------------|------------|-------------------------------|--------|--------|
|             |               |            | 0.005                         | 0.01   | 0.02   |
| TE x PB→TE  | Discontinuous | SJRP       | 16.9*                         | 32.5** | 7.3    |
|             |               | GO         | 6.7                           | 19.1** | 11.1   |
|             | Continuous    | SJRP       | 6.7                           | 32.0** | 14.0** |
|             |               | GO         | 8.0                           | 23.3** | 16.4*  |

*Table 3.* Mortality differences in percentage in the comparison between PB discontinuous and continuous treatments for both SJRP and GO populations when exposed to temephos. Absence of significant differences.

| Comparisons                           | Origin | Temephos concentration (mg/l) |      |      |
|---------------------------------------|--------|-------------------------------|------|------|
|                                       |        | 0.005                         | 0.01 | 0.02 |
| Discontinuous x Continuous<br>(PB→TE) | SJRP   | 7.8                           | 6.5  | 1.2  |
|                                       | GO     | 6.5                           | 14.0 | 6.1  |

*Table 4.* Mortality differences in percentage and its significance, in the comparison between PB pretreated and not treated SJRP and GO populations when exposed to temephos. \*=  $P < 0.05$ ; \*\*=  $P < 0.01$

| Comparisons | Treatment     | Test  | Temephos concentration (mg/l) |        |        |
|-------------|---------------|-------|-------------------------------|--------|--------|
|             |               |       | 0.005                         | 0.01   | 0.02   |
| SJRP x GO   | Discontinuous | TE    | 37.8**                        | 23.6** | 12.3*  |
|             |               | PB→TE | 27.6**                        | 10.2   | 16.1*  |
|             | Continuous    | TE    | 25.0**                        | 39.4** | 18.6** |
|             |               | PB→TE | 26.3**                        | 30.7** | 21.0*  |



Table 5. Presence and frequency (%) of esterase bands, in different developmental stages, of SJRP population submitted to continuous and discontinuous PB pretreatments (T) and controls (C). L4= fourth instar larvae; P= pupae; F= adult females; M= adult males; N= number of analyzed mosquitoes.

|        | (C) Discontinuous |      |      |      | (T) Discontinuous |      |      |      | (C) Continuous |      |      |      | (T) Continuous |      |      |      |
|--------|-------------------|------|------|------|-------------------|------|------|------|----------------|------|------|------|----------------|------|------|------|
|        | L4                | P    | F    | M    | L4                | P    | F    | M    | L4             | P    | F    | M    | L4             | P    | F    | M    |
| N      | 60                | 50   | 28   | 32   | 60                | 50   | 28   | 32   | 63             | 67   | 39   | 39   | 73             | 67   | 41   | 41   |
| Bands  |                   |      |      |      |                   |      |      |      |                |      |      |      |                |      |      |      |
| EST-21 | 3.3               | -    | -    | -    | 1.7               | -    | -    | -    | -              | -    | -    | -    | -              | -    | -    | -    |
| EST-20 | 80.0              | -    | -    | -    | 58.3              | -    | -    | -    | 82.5           | -    | -    | -    | 58.9           | -    | -    | -    |
| EST-19 | -                 | -    | -    | -    | -                 | -    | -    | -    | 11.1           | -    | -    | -    | 4.1            | -    | -    | -    |
| EST-18 | -                 | -    | -    | -    | -                 | -    | -    | -    | 7.9            | -    | -    | -    | -              | -    | -    | -    |
| EST-16 | 1.7               | -    | -    | -    | -                 | -    | -    | -    | -              | -    | -    | -    | 9.5            | -    | -    | -    |
| EST-14 | 33.3              | 48.0 | 46.4 | 31.2 | 40.0              | 36.0 | 32.1 | 28.1 | 36.5           | 59.7 | 35.9 | 35.9 | 61.6           | 76.1 | 46.3 | 26.8 |
| EST-13 | 41.7              | 36.0 | 7.1  | 6.2  | 43.3              | 48.0 | 14.3 | 3.1  | 39.7           | 37.3 | 35.9 | 38.5 | 34.2           | 26.9 | 43.9 | 34.1 |
| EST-12 | 20.0              | 8.0  | -    | -    | 18.3              | 18.0 | -    | -    | 9.5            | 10.4 | 12.8 | 17.9 | 13.7           | 6.0  | 14.6 | 17.1 |
| EST-8  | 20.0              | -    | -    | -    | 8.3               | -    | -    | -    | 7.9            | -    | -    | -    | -              | -    | -    | -    |
| EST-7  | 55.0              | 50.0 | -    | -    | 55.0              | 44.0 | -    | -    | 22.2           | 53.7 | -    | 53.8 | 30.1           | 55.2 | -    | 53.6 |
| EST-6  | 55.0              | 18.0 | 7.1  | -    | 56.7              | 16.0 | 3.6  | -    | 50.8           | 22.4 | -    | 2.6  | 49.3           | 19.4 | -    | 2.4  |
| EST-5  | 31.7              | -    | -    | -    | 20.0              | -    | -    | -    | 15.9           | 7.5  | -    | -    | 20.5           | 3.0  | -    | -    |
| EST-4  | 73.3              | 20.0 | -    | -    | 71.7              | 20.0 | -    | -    | 87.3           | 3.0  | 10.3 | -    | 65.7           | 3.0  | 4.9  | -    |
| EST-3  | 30.0              | -    | -    | -    | 38.3              | 2.0  | -    | -    | 41.3           | 1.5  | -    | -    | 28.8           | 3.0  | 4.9  | 9.8  |
| EST-2  | 38.3              | -    | -    | -    | 21.7              | -    | -    | -    | 46.0           | -    | -    | -    | 23.3           | -    | -    | -    |
| EST-1  | 100               | 100  | 100  | 100  | 100               | 100  | 100  | 100  | 100            | 100  | 100  | 100  | 100            | 100  | 100  | 100  |
| EST-A  | -                 | -    | -    | -    | 1.7               | 2.0  | -    | -    | -              | 4.5  | -    | -    | -              | -    | -    | -    |
| EST-B  | 10.0              | -    | -    | -    | 11.7              | -    | -    | -    | 20.6           | 3.0  | -    | -    | 17.8           | 3.0  | -    | -    |
| EST-C  | 13.3              | -    | -    | -    | 10.0              | -    | -    | -    | 17.5           | 1.5  | -    | -    | 8.2            | 4.5  | -    | -    |
| EST-D  | 10.0              | 2.0  | -    | -    | 10.0              | 4.0  | -    | -    | 6.3            | 6.0  | -    | -    | 1.4            | 3.0  | -    | -    |

- = absence of bands



*Table 6.* Bands that showed significant frequency increase or decrease in the comparisons between control and PB pretreated mosquitoes from SJRP.  
 \* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; > = frequency increase; < = frequency decrease.

| Discontinuous |       |       |       | Continuous  |            |       |       |
|---------------|-------|-------|-------|-------------|------------|-------|-------|
| L4 x L4       | P x P | F x F | M x M | L4 x L4     | P x P      | F x F | M x M |
| EST-20 < *    | -     | -     | -     | EST-20 < ** | -          | -     | -     |
| -             | -     | -     | -     | EST-14 > ** | EST-14 > * | -     | -     |
| -             | -     | -     | -     | EST-4 < **  | -          | -     | -     |
| EST-2 < *     | -     | -     | -     | EST-2 < **  | -          | -     | -     |

- = absence of significant differences



*Table 7.* Mean values and standard deviation in pixels obtained by Image Analysis of the esterase bands EST-1, EST-13 and EST-14, in gels prepared with control and PB pretreated mosquitoes from SJRP. N= number of individuals whose bands were measured. Significance of the comparison with control: \*=  $P < 0.05$

| Bands  | Test | N  | Gray values<br>$\bar{X} \pm S_{\bar{X}}$ |
|--------|------|----|------------------------------------------|
| EST-1  | T    | 67 | 76.7 $\pm$ 3.9*                          |
|        | C    | 67 | 56.2 $\pm$ 4.3                           |
| EST-13 | T    | 18 | 174.2 $\pm$ 9.7*                         |
|        | C    | 26 | 194.1 $\pm$ 4.2                          |
| EST-14 | T    | 51 | 159.1 $\pm$ 5.8                          |
|        | C    | 40 | 165.3 $\pm$ 6.0                          |

*Table 8.* L4 mortality differences in percentage and its significance in tests involving the use of piperonyl butoxide (PBO), phenobarbital (PB) and temephos (TE). The arrow indicates the sequence of treatment with the drugs. += simultaneous treatment; N= number of larvae used; M= mortality percentage. \*=  $P<0.05$ ; \*\*=  $P<0.01$ .

| Experiment        | Test      | N  | M (%)  |
|-------------------|-----------|----|--------|
| not PB pretreated | TE        | 14 | 50.0   |
|                   | PBO→TE    | 14 | 57.5   |
| PB pretreated     | PB→TE     | 30 | 40.0** |
|                   | PB→PBO+TE | 30 | 6.7    |
| PB pretreated     | PB→TE     | 40 | 52.5*  |
|                   | PB→PBO→TE | 50 | 32.0   |



## Discussion

Resistance to insecticides is today one of the most important subjects when insects vectors of diseases and agricultural pests are involved. Some years ago, phenobarbital was shown to promote changes in the insecticide resistance degree increasing tolerance, in rodents and flies, thus becoming an important tool in the study of resistance mechanisms. It was then considered interesting to use it in the study of resistance in such an important vector as *Aedes aegypti*.

In this work, PB was basically used in *A. aegypti* to find out if it works as observed previously in the other mentioned organisms and also to see if it has some effect on esterase patterns.

The data obtained in the comparisons of PB pretreated mosquitoes followed by temephos bioassay are indicative that PB acts in *A. aegypti* as observed in the previous studies for rodents and flies: it increases the insecticide tolerance, in this case, to the temephos. The resistance induction by PB was more efficient at 0.01mg/l concentration, which supplied higher levels of resistance with significant differences in every test. At 0.01mg/l, both treatments (discontinuous, for 30 hours, and continuous, from egg to larval stage) showed the same efficiency in providing protection against temephos effect. However some of these treatments were also significantly efficient using temephos at 0.005mg/l (discontinuous) or 0.02mg/l (continuous), the first concentration being efficient only for mosquitoes from SJRP.

Literature shows that most compounds that act as resistance inducers are effective only at relatively high levels. With many plant compounds this occurs at

about 0.1%-1% in the food (Terriere, 1984). In other cases, some effects also occur at lower levels. For example, *Helicoverpa armigera*, a common pest of many crops in Australia and other countries, has developed resistance to some insecticides. Among them, pyrethroid permethrin that, added to the diet, is already a little effective at levels as low as 0.001%. At concentrations up to 0.005% the degree of induction is greater, but the levels of mortality are also very higher (Xiao-Ping & Hobbs, 1995).

In the present study, PB was used in a single concentration, but the time of treatment varied: thirty hours (discontinuous treatment) or about one week, from egg to L4 (continuous treatment). The results indicated that the continuous treatment was more effective since in both populations the mortality decrease was significantly higher than that in not pretreated mosquitoes and also higher than in the discontinuous treatment although the difference was not significant.

Using *Drosophila melanogaster* treated with different concentrations of PB, Fuchs, Spiegelman and Belitsky (1994) observed that the induction effect was dose dependent and exhibited a significant increase of up to 2.5 times when compared with the controls. The activity was evaluated by inducibility of cytochrome P-450 isozymes.

The results in SJRP and GO, without pretreatment, showed that larvae from SJRP are more sensitive to the temephos than larvae from GO, reinforcing previous data indicating that this population is resistant to insecticides (Macoris et al., 1995).

As to the efficiency of PB in providing protection, both populations in the two types of experiments (discontinuous and continuous treatments) showed



mortality decrease caused by this substance. However, the reduction was greater for SJRP than for GO and greater at the 0.01mg/l than for the other concentration of temephos. Therefore, the mosquitoes from SJRP responded with greater intensity to the PB treatment. It is also interesting to remember that at 0.005mg/l only SJRP showed significant decrease in mortality. Sousa-Polezzi and Bicudo (b, to be published elsewhere) also observed that the developmental time and longevity were more affected by PB in SJRP than in GO.

The phenomenon of insecticide resistant strains being less sensitive to PB induction compared to susceptible strains has been noted previously in some studies (Yu & Terriere, 1973; Hällström, Blanck & Atuma, 1984; Lee & Scott, 1989; Scott & Lee, 1993; Fuchs, Spiegelman & Belitsky, 1994). According to Fuchs, Spiegelman and Belitsky (1994), the PB molecule by itself, but not by its derivatives, induces cytochrome isozymes that are considered important in providing insecticide detoxification. They also said that the lower effect in resistant strains suggests that PB fails to induce this system in them due to the enhanced PB degradation capacity by their high basal level of cytochrome isozymes.

In the search for differences between the control and the PB treated larvae, the esterase enzymes were analyzed. In the discontinuous treatment two bands showed frequency decrease, EST-2 and EST-20. In the continuous treatment, besides these two, a third band, EST-4, also showed frequency decrease. In the continuous but not in the discontinuous treatment, another band, EST-14, exhibited frequency increase. Increased activity degree indicated by greater staining intensity and thickness was observed in bands EST-13 and EST-14



(considered to be alleles from the same locus by Lima-Catelani, Bicudo & Ceron, to be published elsewhere), but decreased activity occurred in the band EST-1. These observations were more evident in mosquitoes submitted to the continuous treatment.

Because the present results of continuous and discontinuous treatments, followed by the exposition to the insecticide temephos, showed that PB caused increase of the resistance, we can infer that the bands EST-2, EST-4 and EST-20 (that showed frequency reduction in the treatments) are not involved in the tolerance induced by PB, but they can be related with the problems caused in the development of the treated mosquitoes (Sousa-Polezzi & Bicudo b, to be published elsewhere). But EST-14, with significant increase of frequency and EST-13 with increased activity in the continuous treatment, might be performing some role in the increase of tolerance to temephos. These enzymes are included in the group of cholinesterases, that have been considered involved in the insect resistance to organophosphorous insecticides (Smitsaert, 1964; Devonshire, 1975; Fournier & Mutero, 1994; Baxter & Barker, 1998; Zhu & Gao, 1999).

On the other hand, we should remember that, according to some literature data for other organisms, the resistance to PB is considered to occur due to cytochrome oxidases action (Carino et al., 1994; Brun et al., 1996; Liu & Scott, 1998) and then, in *Aedes*, esterases would have a secondary role. P-450-dependent mixed function oxidases can metabolize insecticides present in all living organisms (Bourguet, Capela & Raymond, 1996) and have been considered more important in resistance to pyrethroids than to organophosphorous insecticides (Bisset et al., 2000).





In the study of the effect of the inducer phenobarbital on susceptible and resistant strains of *Musca domestica*, Moldenke and Terriere (1981) obtained evidence that the various forms of P-450 were produced in different amounts in treatments with the inducer, so that a form barely detectable in the susceptible flies became rather prominent on induction. In this case, a strain or species could also possess inactive genes for a certain enzyme and such genes would be activated in the presence of an inducing agent, thereby aiding in its metabolism (Terriere, 1984).

The use of piperonyl butoxide (PBO) is a common procedure to determine whether cytochrome P-450 is involved in the insecticide resistance. PBO inhibits mixed functions oxidases and subsequently reduces the number of molecules available to degrade or activate (or both) the insecticide. Consequently it reduces the resistance (Oppenoorth & Welling, 1976 apud Xiao-Ping & Hobbs, 1995; Bourguet, Capela & Raymond, 1996).

We used a bioassay in which *A. aegypti* larvae from SJRP were preexposed to PBO and after to temephos. Resistance rates were lower when PBO was used, but the difference was not significant. In this case, PBO might be acting at least in a small degree and thus oxidases might explain part of the insecticide resistance observed.

In order to verify if oxidases are involved in the resistance induced by PB (inductor) larvae PB pretreated were exposed to PBO, before or simultaneously with the temephos. The experiments, in both cases showed opposite results, relative to the previous one (using only PBO), that is, significantly lower mortality rates (or higher level of resistance) occurred when PB pretreated larvae were

submitted to PBO treatment. In this case both substances are apparently acting in synergism, that is, PBO is reinforcing the effect of PB, and thus, presumably oxidases are not responsible for resistance induced by PB in *Aedes*, contrarily to what was found in *Drosophila* (Brun et al., 1996; Dombrowski et al., 1998) and *Musca domestica* (Liu & Scott, 1997). It is interesting that Wirth (1998), using PBO found that oxidases are not involved in organophosphate resistance in *Culex pipiens*. Two highly active esterases or one expressing insensitive acetylcholinesterase were the mechanisms considered responsible for resistance in two strains of that organism. These observations in *Culex* reinforce that the main mechanism acting in insecticide resistance observed in *A. aegypti* in the present study is related to the esterase bands EST-13 and EST-14. It also suggests that mosquitoes *Culex* and *Aedes* might share this mechanism.

The present results support the idea that resistance is a complex answer of the organisms, involving activation of several regulatory pathways, probably with predominance of any of them depending on the inducer agent, on the organism and their genotype and also on the environmental circumstances.



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## VI. DISCUSSÃO GERAL

No Brasil, as pesquisas e, conseqüentemente, o conhecimento sobre resistência a inseticidas de vetores e outras pragas ainda são bastante incipientes. Isto ocorre, apesar da relevância do problema, decorre de que o principal processo de controle desses organismos é pelo uso das referidas substâncias. Em geral, a maioria das pesquisas nesta área são iniciadas somente após a detecção de dificuldades nos programas de controle causadas pela resistência ao inseticida em uso. Sabe-se, porém, que os programas de manejo da resistência são mais efetivos quando implementados de modo preventivo, ou seja, antes da sua evolução (Georghiou, 1994; Roush & Miller, 1986).

Como já foi mencionado neste trabalho, o aumento da síntese de esterases está relacionado com a resistência a inseticidas, conforme foi constatado em várias espécies de mosquitos e outros organismos. Em populações de *Aedes aegypti* resistentes a organofosforados, a literatura também mostra níveis aumentados de atividade esterásica (Mourya *et al.*, 1993; Mazzarri & Georghiou, 1995; Vaughan *et al.*, 1998).

Assim, no presente trabalho, procuramos estudar aspectos da resistência a inseticidas relacionados com alterações no padrão de esterases de *A. aegypti*. O primeiro desses estudos envolveu a comparação dos tipos e frequências das bandas esterásicas detectadas presentemente na população de *A. aegypti* de SJRP, com as mesmas características observadas nessa população, em análise realizada de 1993 a 95 (Lima-Catelani, 1996). Nessa comparação, foram constatadas alterações quantitativas e qualitativas entre as duas análises. Entre as bandas que



apresentaram alteração de frequência, oito tiveram redução significativa e duas apresentaram aumento, em diferentes fases do desenvolvimento. Destacam-se EST-12 e EST-14 que apresentaram redução de frequência em todas as fases, exceto na pupa, para a EST-14 e nas fêmeas adultas, para a EST-12. As bandas que sofreram aumento foram EST-2 e EST-7.

No que se refere a essas alterações de frequência, ocorridas no espaço de tempo entre as duas análises de SJRP, é evidente que não se pode afirmar que todas estejam relacionadas com mudanças no grau de resistência. Entretanto, dado que as esterases que sofreram alteração, em sua maioria se classificam como colinesterases ou carboxilesterases, sabidamente envolvidas com resistência a inseticidas, em vários organismos já estudados, coloca-se essa possibilidade com uma certa ênfase. Mesmo as bandas que sofreram redução de frequência podem ter sido substituídas por outras, geneticamente diferenciadas, dotadas de melhor desempenho para esse fim, isto é, o aumento da resistência.

Com relação às diferenças qualitativas, na análise mais recente da população de SJRP verificamos a presença de um conjunto de quatro bandas  $\alpha\beta$  esterásicas (EST-A, EST-B, EST-C e EST-D), localizadas na região menos anódica do gel, não detectadas no estudo apresentado por Lima-Catelani (1996), para a mesma população. Tendo em vista o tempo decorrido desde aquele trabalho, e considerando que durante todo esse tempo houve sucessivas pulverizações de inseticidas pela SUCEN, para controle dos mosquitos adultos, inicialmente com organofosforados e depois com piretróides, e ainda, o constante uso de granulados organofosforados para controle da fase larval, aventamos a hipótese de que a presença dessas bandas constitua uma resposta àqueles



tratamentos. Como já mencionado, estudos citogenéticos na população de *A. aegypti* de SJRP revelaram a presença de altas frequências de aberrações cromossômicas que poderiam estar relacionadas ao uso freqüente de inseticidas (Lima-Catelani & Bicudo, 1994).

As novas bandas  $\alpha\beta$  esterásicas, classificadas nos testes de inibição que realizamos, como colinesterases, também foram detectadas na população resistente de Goiânia com frequências maiores do que em SJRP, reforçando a hipótese de que essas colinesterases têm função de resistência. Além disso, a ausência dessas bandas na análise de Lima-Catelani (1996) nos mosquitos de SJRP e sua presença neste estudo, com frequências mais baixas do que as apresentadas na população de GO, sugerem que os mosquitos portadores dessas esterases estão sendo selecionados e reitera a idéia de que a população de SJRP está se tornando resistente. É interessante ressaltar que essas bandas apresentaram frequências mais altas em larvas, nas duas populações estudadas, que é exatamente o estágio submetido ao controle com o inseticida temephos, sugerindo que a seleção e ativação gênicas estão ocorrendo nessa fase do desenvolvimento. De acordo com Georghiou & Taylor (1977), a seleção é um dos processos capazes de influenciar a evolução da resistência quando um inseticida já tem utilização generalizada na área.

Outra esterase que também parece estar sendo selecionada, em larvas, é a EST-9. Esta banda também mostrou aumento de frequência na população de SJRP, entre o estudo de Lima-Catelani (1996) (24,7%) e o presente estudo (52,3%). Na população de GO essa frequência foi ainda maior (77,8%). Todos os aumentos foram significantes.





Nas duas populações estudadas (SJRP e GO), paralelamente à redução de frequência no estágio larval, as esterases EST-3 e EST-5 apresentaram ativação gênica em outras fases do desenvolvimento. Já a EST-2 aumentou significativamente sua frequência no estágio larval quando comparada com a análise de Lima-Catelani (1996) (de 6,8% para 33,8% em SJRP e 36,5% em GO). Esta banda foi considerada, por aquela autora, como possivelmente envolvida na resistência. Os presentes resultados reforçam essa indicação.

No presente trabalho, a banda EST-1 foi completamente inibida pelo PMSF que é um reconhecido inibidor de serino-proteases, podendo também atuar da mesma forma em outras hidrolases. Segundo Lima-Catelani (1996), a banda EST-1 pode corresponder à EST-6, reconhecida como envolvida na resistência em *Drosophila*. A banda EST-1, dotada de intensa coloração e espessura, foi encontrada em todas as fases do desenvolvimento de *A. aegypti*, indicando desempenhar um papel fisiológico importante. Os resultados das duas análises de SJRP sugerem que EST-1 e EST-2 são alelos do mesmo *locus*.

A observação de aumento de frequência de algumas bandas e diminuição ou mesmo ausência de outras, previamente detectadas na população de SJRP, pode sugerir uma substituição de alelos sob pressão ambiental, na qual os inseticidas exercem importante efeito. Várias dessas mudanças observadas em mosquitos de SJRP tornam essa população mais semelhante à GO, como verificado pela análise do índice de similaridade. Assim as múltiplas alterações no padrão de esterases, que incluem seleção e regulação gênica diferencial durante o desenvolvimento, estão ocorrendo em GO e SJRP e podem estar relacionadas ao uso constante de inseticidas.

O uso contínuo de um determinado inseticida pode elevar a frequência de alelos envolvidos na resistência em uma população para níveis em que a eficácia do produto é comprometida. Os principais processos evolutivos determinantes da frequência de alelos resistentes em uma população, antes da introdução de um inseticida na área, são mutação, seleção, fluxo gênico e deriva genética. Contudo, seleção e fluxo gênico passam a ser os processos mais importantes, capazes de influenciar a evolução da resistência, quando um inseticida já tem utilização generalizada na área (Georghiou & Taylor, 1977). Dentre os fatores mais relevantes, que atuam diferencialmente sobre os processos evolutivos na resistência, estão a frequência inicial dos alelos resistentes, a pressão de seleção para a resistência, a dominância funcional do(s) alelo(s) resistentes(s), o valor adaptativo de heterozigotos e homozigotos, a diluição da resistência por migração e o número de gerações por ano (Georghiou & Taylor, 1977).

Outro aspecto abordado neste trabalho foi o estudo do efeito do fenobarbital (PB), um barbitúrico com propriedades anticonvulsivas. Vários autores verificaram que essa droga é capaz de aumentar a resistência em roedores (Clement, 1983; Kaliste-Korhonen *et al.*, 1990) e moscas (Scott & Lee, 1993; Fuchs *et al.*, 1994). Esta capacidade tornou a referida droga uma importante ferramenta no estudo dos mecanismos de resistência. Acredita-se que esta proteção contra a toxicidade, conferida pelo PB, resulte da indução de hidrolases, carboxilesterases ou oxidases (Kaliste-Korhonen *et al.*, 1990; McCracken *et al.*, 1993). Foram objetivos básicos do presente trabalho verificar se a resistência aumentada, causada por essa substância em outros organismos, ocorreria também em *Aedes* e, ainda, se causaria alterações no padrão de síntese das esterases. Duas

populações de *A. aegypti* foram escolhidas para o estudo, SJRP e GO, esta última considerada resistente por Macoris *et al.* (1995).

A análise dos efeitos do PB no padrão de esterases e na resistência foi precedida pela análise de seus efeitos no desenvolvimento do mosquito. Essa análise foi indicativa de alterações ocorridas durante o desenvolvimento. O tratamento não afetou a taxa de eclosão, mas produziu um atraso no tempo de desenvolvimento, que foi maior para larvas de 4º estágio e pupas.

Embora o atraso no desenvolvimento tenha ocorrido para as duas populações, as diferenças entre controle e tratados foram maiores em SJRP do que em GO, o inverso acontecendo em relação à longevidade. Admitiu-se que esta diferença pode ser devida ao maior nível de resistência em GO. Fuchs *et al.* (1994) haviam observado que linhagens resistentes e suscetíveis de *Drosophila* respondem diferentemente ao tratamento com PB. As resistentes mostraram menor inducibilidade do sistema citocromo P450. De acordo com os autores mencionados isto é devido a uma degradação de PB maior nos insetos resistentes.

O efeito mais marcante foi observado no final do estágio de pupa, em que uma porcentagem relativamente alta de imagos morreu durante a emergência impossibilitados de sair do casulo. Até mesmo quando cuidadosamente retirados, esses imagos morreram em menos de 24 horas. Esta observação somada à lentidão de movimentos dos mosquitos tratados sugere alterações no sistema nervoso. Como já mencionado, vários estudos indicaram efeitos negativos do PB no desenvolvimento desse sistema como, por exemplo, a redução na quantidade de neurônios (Loefering & Johnson, 1994) e o alto índice apoptótico em cérebro de rato recém-nascido (Ikonomidou *et al.*, 2000).





Além disso, a literatura mostra que agentes que potencializam o ácido  $\gamma$ -aminobutírico (GABA) (como é o caso do PB) ou que diminuem os seus níveis (tais como alilglicina) afetam o desenvolvimento cerebral. Larvas de *D. melanogaster*, alimentadas com alilglicina, empuparam e se desenvolveram normalmente, mas os imagos foram incapazes de emergir (Howard *et al.*, 1975). De acordo com esses autores, a alilglicina provavelmente afeta algum processo vital para adultos que não é essencial para larvas.

Assim, o atraso no desenvolvimento, a impossibilidade para emergir do casulo pupal e a menor longevidade dos adultos, indicam que importantes processos biológicos estão sendo afetados nos mosquitos tratados com PB. A observação, em outros organismos inclusive o homem, de que o tratamento a longo prazo com PB tem diversos efeitos prejudiciais, é suportada pelos presentes dados.

Testes para avaliação da resistência ao temephos sem qualquer tratamento prévio das amostras evidenciaram maior grau de resistência na população de GO quando comparada com SJRP. Esses dados confirmaram os obtidos por Macoris *et al.* (1995) quanto à resistência apresentada pela população de GO.

Quanto ao efeito do PB no aumento de tolerância ao organofosforado temephos, a análise da mortalidade em larvas pré-tratadas e expostas a esse inseticida indicou que aquela substância agiu como observado em estudos anteriores, ou seja, houve redução da mortalidade nas duas populações. Essa redução foi maior na concentração de 0,01mg/L de temephos, para ambos os tratamentos utilizados, descontínuo e contínuo. Na concentração de 0,02mg/L



houve diferença significativa apenas para o tratamento contínuo, indicando que a exposição prolongada é mais efetiva na indução de tolerância ao PB.

Quanto à comparação da eficiência do PB entre as duas populações, observamos que SJRP respondeu com maior intensidade, aumentando a resistência em níveis superiores aos de GO. É interessante lembrar que, na concentração de 0.005mg/L, somente SJRP mostrou redução significativa da mortalidade. A observação de que a linhagem susceptível mostrou-se mais sensível à indução pelo PB do que a considerada resistente está de acordo com outros estudos (Yu & Terriere, 1973; Hällström *et al.*, 1984; Scott & Lee, 1993; Fuchs *et al.*, 1994).

A análise de mudanças dos padrões de esterases, sob efeito do PB, foi realizada pela comparação entre controles e tratados. No tratamento descontínuo, as bandas EST-2 e EST-20 mostraram diminuição significativa de frequência. No tratamento contínuo, além dessas, a EST-4 também apresentou diminuição de frequência e a EST-14, aumento. Quanto ao nível de atividade, EST-13 e EST-14 foram as bandas que apresentaram maior intensidade de coloração e maior espessura nas pupas tratadas, enquanto EST-1 mostrou redução dessas características, indicando diminuição de atividade. Essas observações foram mais evidentes nos mosquitos submetidos ao tratamento contínuo.

Como os resultados dos tratamentos contínuo e descontínuo, seguidos da exposição ao inseticida temephos, mostraram que o PB causou aumento da resistência, pode-se inferir que as bandas EST-2, EST-4 e EST-20, que sofreram redução de frequência nos tratamentos, não estão envolvidas na resistência induzida por PB. Contudo, podem estar relacionadas com os problemas causados

no desenvolvimento dos mosquitos tratados com essa substância. Já as bandas EST-14, com frequência significativamente maior, e a EST-13 com atividade elevada no tratamento contínuo, podem estar desempenhando algum papel na resistência aumentada ao temephos. Estas enzimas pertencem ao grupo das colinesterases, sabidamente envolvidas na resistência a inseticidas organofosforados (Smissaert, 1964; Devonshire, 1975; Fournier & Mutero, 1994; Baxter *et al.*, 1998; Zhu & Gao, 1999) e podem ser consideradas como alelos do mesmo *locus*, pelos dados obtidos no presente trabalho.

Os efeitos do temephos sobre os padrões de esterases também foram estudados, verificando-se, em geral, que o mesmo é de inibição. Em larvas submetidas ao tratamento com esse inseticida, sozinho ou após pré-tratamento com PB, ocorre inibição das bandas EST-3 a EST-6 e EST-18, nos mosquitos das duas localidades, e inibição parcial das bandas novas EST-A a EST-D e de EST-7, EST-12 a EST-14 e EST-20. Apenas EST-1 foi preservada na presença do inseticida temephos, apesar de sofrer inibição significativa após tratamento com PB. Essa banda, como já foi mencionado, ocorre em 100% dos indivíduos analisados, tanto por Lima-Catelani (1996) como no presente estudo, sugerindo que ela é importante para a sobrevivência.

O uso do butóxido de piperonila (PBO) é um procedimento comum para determinar se as oxidases são responsáveis pela resistência a inseticidas, em uma determinada população. De acordo com dados da literatura, a redução de mortalidade conferida pelo PB pode ocorrer pela indução de oxidases, como demonstrado em *D. melanogaster* e *Musca domestica* (Carino *et al.*, 1994; Brun *et al.*, 1996; Liu & Scott, 1998). PBO inibe as oxidases e, conseqüentemente,

reduz o número de moléculas disponíveis para degradar e ou ativar o inseticida, o que causa aumento da mortalidade (Oppennorth & Welling, 1976 apud Xiao-Ping & Hobbs, 1995; Bourguet *et al.*, 1996). As oxidases podem metabolizar inseticidas e têm sido consideradas mais importantes na resistência a piretróides do que a organofosforados (Bisset *et al.*, 2000).

No presente estudo, utilizou-se o PBO para verificar se as oxidases estão envolvidas na resistência de *A. aegypti*, em amostras de SJRP. A taxa de mortalidade foi maior quando PBO foi utilizado, mas não em nível significativo, sugerindo que as oxidases não desempenham papel relevante na resistência observada.

Para verificar se as oxidases estão envolvidas na ação do fenobarbital, larvas pré-tratadas com essa substância foram também submetidas ao PBO, antes ou simultaneamente à exposição ao temephos. Os experimentos, em ambos os casos, mostraram resultados opostos aos obtidos sem pré-tratamento com PB, ou seja, a resistência foi maior para larvas tratadas com PBO do que para larvas não tratadas. Neste caso, as duas substâncias parecem estar atuando em sinergismo, ou seja, o PBO está acentuando o efeito do PB. Assim, possivelmente as oxidases não são responsáveis pelo aumento da tolerância conferida pelo PB em *A. aegypti*, o que reforça o papel das colinesterases (EST-13 e EST-14), encontradas com maior frequência e atividade no presente estudo, após tratamento com PB.

De maneira geral, os resultados do presente estudo suportam a idéia de que a resistência é uma resposta complexa dos organismos, envolvendo seleção e ativação de várias vias regulatórias. Uma ou outra dessas vias pode predominar,





dependendo do agente indutor, do organismo ou, até mesmo, do genótipo dos indivíduos e de outras circunstâncias ambientais, além da presença de inseticidas.

As informações decorrentes deste estudo constituem um primeiro passo para a elucidação dos mecanismos envolvidos no desenvolvimento de resistência em populações brasileiras do vetor *A. aegypti*, indicando a necessidade do início de um programa de monitoramento da resistência e aplicação de outras medidas alternativas de controle além do uso de inseticidas.





## VII. CONCLUSÕES

1. A comparação dos padrões de esterases observados no presente estudo da população de SJRP com os observados em análise anterior da mesma população bem como a comparação entre os padrões de SJRP e GO mostraram a presença de quatro novas bandas nas duas localidades (com maior frequência em GO), indicando relação com o desenvolvimento de resistência ao inseticida temephos, em larvas.
2. As mesmas comparações mostraram também alterações de frequência, em SJRP, de bandas anteriormente descritas, destacando-se aumento de frequência de EST-2, no estágio larval, e EST-7, nas pupas e machos adultos e a redução de frequência das bandas EST3 a EST5, EST-7, EST-12, EST-14 e EST-20, no estágio larval. Essas observações sugerem a possibilidade de substituição de alelos sob pressão ambiental e, também, que o desenvolvimento de resistência nas populações analisadas pode ser devido à soma de várias modificações em diferentes *loci* esterásicos e não decorrente da atividade de um único *locus*.
3. A análise de similaridade entre as populações, considerando a presença e a frequência das bandas, mostrou que a população atual de mosquitos de SJRP assemelha-se mais à população resistente de GO do que à população de SJRP na análise anterior;



4. Fenobarbital (PB) foi administrado aos mosquitos com o objetivo básico de verificar se essa substância reduz a mortalidade causada pelo inseticida temephos, nesses organismos. O tratamento contínuo com PB causou atraso no desenvolvimento, diminuição na longevidade dos adultos, afetando também a emergência dos imagos, no estágio de pupa (30% dos imagos não conseguiram emergir do casulo). Algumas observações sugerem que o PB afeta o sistema nervoso de *Aedes aegypti*, reforçando resultados previamente obtidos em outros organismos.
5. O pré-tratamento com PB resultou no aumento de tolerância ao temephos, em *Aedes aegypti* nas duas populações analisadas, mostrando que esse organismo responde a essa substância como observado, anteriormente, em roedores e moscas.
6. Tanto o pré-tratamento contínuo como o descontínuo, com PB, causaram diminuição significativa da mortalidade nas larvas, na concentração de 0,01mg/L de temephos. Na concentração de 0,02mg/L de temephos, porém, somente o tratamento contínuo mostrou níveis significantes de redução da mortalidade.
7. As taxas de redução da mortalidade em larvas pré-tratadas com PB foram maiores em SJRP do que em GO, o que está de acordo com dados da literatura, segundo os quais, os organismos resistentes reagem menos ao tratamento que os sensíveis.



8. A análise dos efeitos do PB, no padrão de esterases, mostrou que os genes para EST-14 e EST-13, classificadas como colinesterases, e que possivelmente são alelos do mesmo *locus*, podem estar envolvidos na tolerância induzida por essa substância, pelo fato de que essas bandas apresentaram aumento de frequência ou atividade.
9. O butóxido de piperonila (PBO), inibidor de oxidases, utilizado em larvas pré-tratadas com PB indicou que essas enzimas não estão envolvidas no aumento de tolerância e sugeriu que PB e PBO estão atuando em sinergismo para esse fim. O não envolvimento das oxidases reforça o papel das esterases na ação do PB, em *Aedes aegypti*, diferentemente do que foi sugerido para outros organismos.



## VIII. REFERÊNCIAS BIBLIOGRÁFICAS

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## IX. RESUMO

Larvas (L4), pupas e adultos de *Aedes aegypti* de São José do Rio Preto-SP (SJRP) e de Goiânia (GO, considerada resistente a inseticidas), foram analisadas quanto ao padrão de esterases. Os dados obtidos para SJRP foram comparados com os dados obtidos há oito anos, para a mesma população, e também com aqueles obtidos para GO, com o objetivo de detectar possíveis alterações relacionadas com modificações no grau de resistência em SJRP. A observação de quatro novas bandas (colinesterases) em SJRP e GO (em frequências maiores em GO), fortemente indica que essas esterases estão envolvidas no desenvolvimento de resistência na primeira população. O fenobarbital (PB), indutor enzimático em outros organismos, foi utilizado neste estudo mostrando que essa droga causou atraso no desenvolvimento, diminuição da longevidade dos adultos e efeitos no final do estágio de pupa, em que cerca de 30% dos imagos não puderam emergir. O envolvimento do PB na indução de tolerância ao temephos em L4 foi analisado nos tratamentos contínuo (0,2mg/ml, de ovo a L4) e descontínuo (0,26mg/ml, 30 horas), usando três concentrações do inseticida (0,005mg/L, 0,01mg/L e 0,02mg/L). Observou-se diminuição significativa da mortalidade em larvas pré-tratadas com PB, nos dois tratamentos, na concentração de 0,01mg/L de temephos, mas somente o tratamento contínuo mostrou níveis significantes em 0,02mg/L do inseticida. As taxas de redução da mortalidade foram maiores em SJRP do que GO. A análise dos efeitos do PB, no padrão de esterases, mostrou que o *locus* contendo genes para EST-14 (colinesterase) e para EST-13 (possivelmente um alelo do mesmo *locus*), pode estar envolvido na ação do PB. O



butóxido de piperonila (PBO), inibidor de oxidases, utilizado em larvas pré-tratadas com PB indicou que as oxidases não estão envolvidas. Quando larvas pré-tratadas com PB foram submetidas ao PBO e em seguida ao temephos, foram observados níveis mais altos de resistência, sugerindo que PB e PBO atuaram em sinergismo. O não envolvimento das oxidases reforça o papel das esterases na ação do PB. Assim, tanto a resistência "natural", desenvolvida em SJRP, como a tolerância induzida pelo PB parecem estar relacionadas a esterases. Contudo, as observações mostraram que as esterases envolvidas em ambas as situações não são as mesmas.

*Palavras-chaves:* *Aedes aegypti*, esterase, resistência a inseticidas, fenobarbital, temephos.





## X. ABSTRACT

Larvae (L4), pupae and adults of *Aedes aegypti* from São José do Rio Preto-SP (SJRP) and Goiânia (GO, considered resistant to insecticides) were studied as to the esterase patterns. Data on SJRP mosquitoes were compared with data obtained eight years ago for the same population and also with those obtained for GO, aiming to detect possible changes on esterase patterns that could be related to the development of insecticide resistance in SJRP. The finding of four new bands (cholinesterases) in the present study of both SJRP and GO populations (with higher frequencies in GO), strongly indicates that resistance is in progress in the first locality. Phenobarbital (PB), enzyme inducer in other organisms, was used in this study showing a delay in the developmental time, a decrease in adult longevity and effects at the pupal stage, where about 30% of imagoes could not emerge. The involvement of PB in the increase of tolerance to temephos insecticide in L4 was analyzed in continuous (0.2mg/ml of PB, from egg to L4) and discontinuous (0.26mg/ml, 30 hours) treatments, using three concentrations of temephos (0.005mg/l, 0.01mg/l and 0.02mg/l). Significant increased tolerance in PB pretreated larvae occurred in both treatments at 0.01mg/l, but at 0.02mg/l only in the continuous the increase was significant. The mortality reduction rates were greater for SJRP than for GO. Esterase patterns were examined in PB pretreated larvae showing that the locus containing genes for esterase band EST-14 (a cholinesterase) and for band EST-13 (supposed to be an allele of the same locus) is involved in the PB action. Piperonyl butoxide (PBO), that inhibits oxidases, was used in the PB pretreated larvae suggesting that the oxidases are rather not





involved. When PB pretreated larvae were submitted to PBO and later to temephos, increased resistance was observed suggesting that PB and PBO are acting in synergism. The uninvolved of oxidases in the PB induced tolerance, reinforces the role of esterases in the insecticide resistance. Thus, the obtained data indicate that in both the resistance in progress in SJRP and the tolerance induced by PB, esterases are involved. However, the data also showed that the esterases are not the same in the two cases.

*Key Words:* *Aedes aegypti*, esterase, insecticide resistance, phenobarbital, temephos



