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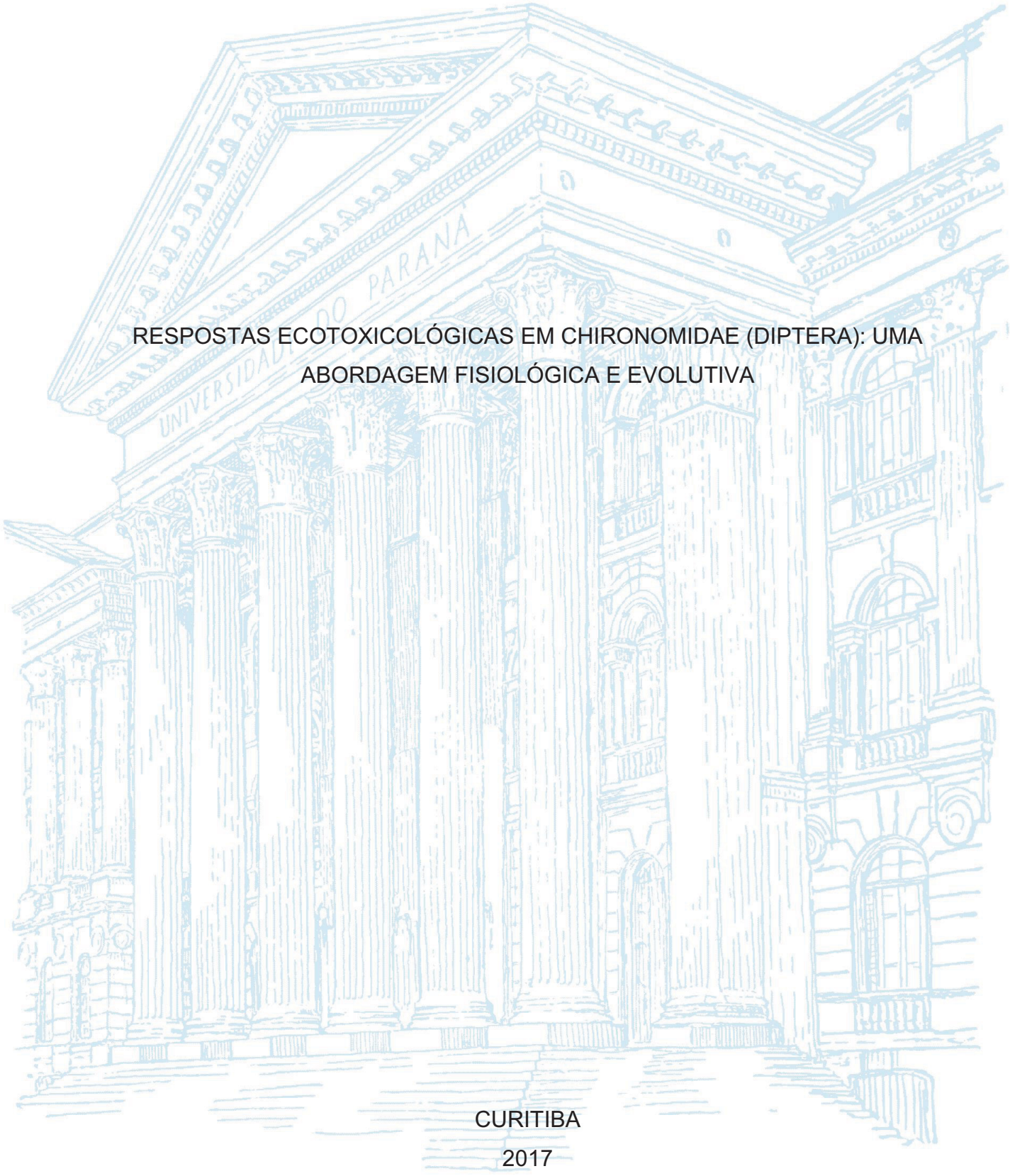

RESPOSTAS ECOTOXICOLÓGICAS EM CHIRONOMIDAE (DIPTERA): UMA ABORDAGEM FISIOLÓGICA E EVOLUTIVA

CURITIBA

2017

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RESPOSTAS ECOTOXICOLÓGICAS EM CHIRONOMIDAE (DIPTERA): UMA ABORDAGEM FISIOLÓGICA E EVOLUTIVA

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas Área de Concentração Entomologia, Departamento de Zoologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial para a obtenção do título de Doutor em Ciências Biológicas (Entomologia).

Orientador: Prof. Dr. Mário Antônio Navarro da Silva
Co-Orientadora: Prof. Dr. Célia Regina Cavichiolo Franco

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VINICIUS SOBRINHO RICHARDI

"RESPOSTAS ECOTOXICOLÓGICAS EM CHIRONOMIDAE (DIPTERA): UMA ABORDAGEM FISIOLÓGICA E EVOLUTIVA"

Tese aprovada como requisito parcial para obtenção do grau de "Doutor em Ciências", no Programa de Pós-graduação em Ciências Biológicas, Área de Concentração em Entomologia, da Universidade Federal do Paraná, pela Comissão formada pelos professores:

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Curitiba, 22 de fevereiro de 2017.

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Com carinho
Vinicius

“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos”.

(Isaac Newton)

RESUMO

A sobrevivência é o maior desafio enfrentado por organismos que habitam ambientes constantemente degradados pela ação antrópica, muitos deles o vencem porém com custos biológicos da exposição. O objetivo da tese foi avaliar as respostas de larvas de Chironomidae sob desafios da sobrevivência em degradação ambiental através de biomarcadores. Primeiramente bioensaios de toxicidade crônica foram realizados em larvas de *Chironomus sancticaroli* expostas a sedimentos provindos de 5 rios e 5 reservatórios do estado de São Paulo, nessas larvas foram avaliados as respostas de biomarcadores como AChE (Acetilcolinesterase), EST-α (Esterase alfa), EST-β (Esterase beta), GST (Glutationa *S*-transferase), CAT (Catalase), SOD (Superóxido-dismutase), LPO (lipoperoxidação) e alterações histológicas. Além disso, larvas de *Chironomus dilutus* foram expostas em um respirômetro por 6 horas a 4 concentrações de arsênio e oxigênio dissolvido e suas combinações resultando em 16 tratamentos. Nesses organismos além do consumo de oxigênio em exposição ao arsênio, foi avaliado para todas as combinações a expressão gênica das hemoglobinas A, B, C, D e a enzima GST. Por fim, 12 espécies distribuídas em *Chironomus, Dicrotendipes* e *Goeldichironomus* foram expostas por 48 horas ao malathion e ao propuxur, nesses organismos foi mensurado a atividade das enzimas AChE, EST-α e EST-β. Com a resposta enzimática foi aplicado as métricas *K* e λ para detecção de sinal filogenético, juntamente com uma filogenia baseada no COI (citocromo oxidase I), além disso respostas de espécies iguais coletadas em locais diferentes foram comparadas para verificar o efeito da pressão de seleção. Para *C. sancticaroli* exposta ao sedimentos, todas as enzimas apresentaram alterações em relação ao grupo controle, além de estresse oxidativo e a histologia demonstrou efeitos principalmente no corpo gorduroso visceral. Em *C. dilutus* houve um aumento no consumo de oxigênio em exposição ao arsênio e aumento na expressão da HbA na exposição ao arsênio, HbB em arsênio e hipóxia, Hb A, D e GST na combinações das duas condições. Nas 13 espécies de Chironomidae expostas a pesticidas, não existe sinal filogenético para a respostas da enzimas, mas para as espécies de *Chironomus* foi observado uma tendência para as enzimas AChE e EST-α. A pressão de seleção se mostrou o principal direcionador das respostas enzimáticas já que mesmas espécies coletadas em locais diferentes, apresentaram níveis diferenciados de atividade. Larvas de Chironomidae possuem uma considerável plasticidade fisiológica que permite-os sobreviver nesse ambientes desafiadores, mesmo sem mortalidade, alterações foram detectadas demonstrando desequilíbrios da homeostase. Mesmo com a pressão de seleção que esses organismos sofrem no ambiente natural, eles se mantém vivos e resistentes, esse quadro pode refletir em algum custo no fitness das espécies a longo prazo.

Palavras-chave: Chironomidae, sedimento, biomarcadores bioquímicos, expressão gênica, biologia comparada

ABSTRACT

Survival is the biggest challenge faced by organisms inhabiting environments constantly degraded by anthropic activities. Several organisms can thrive but they have biological costs of exposure. The aim of the thesis was evaluate the chironomids larvae responses under survival challenges of environmental degradation using biomarkers. First, chronic bioassays were carried out in *Chironomus sancticaroli* larvae exposed to sediments of 5 rivers and 5 reservoirs from São Paulo State. It was measured in these larvae biomarkers as AChE (Acetylcholinesterase), EST-α (alpha-esterase), EST-β (beta-esterase), GST (Glutathione *S*-transferase), CAT (Catalase), SOD (Superoxide-dismutase), LPO (lipid peroxidation) and histopathologies. In addition, *Chironomus dilutus* larvae were exposed in a respirometer for 6 hours to 4 concentrations of arsenic and dissolved oxygen and their combinations resulting in 16 treatments. Beyond oxygen consumption in arsenic exposure, in the combinations were measured gene expression of A, B, C, D hemoglobins and GST enzyme. Finally, 12 species of *Chironomus, Dicrotendipes,* and *Goeldichironomus* were exposed for 48 hours to malathion and propoxur pesticides. The activity of AChE, EST-α e EST-β was measured, and in the results was applied *K* and λ metrics to phylogenetic signal detection also using a phylogeny based in COI gene (cytochrome oxidase I). The responses from same species collected in different sites were compared to verify selection pressure influence. In *C. santicaroli* exposed to sediments, all enzymes showed alterations comparing with control group, also was detected oxidative stress and tissues changes in visceral fat body. In *C. dilutus* there was an increase of oxygen consumption in arsenic exposure, also was observed increase of HbA expression (arsenic exposure), HbB (arsenic and hypoxia) and HbA, D, GST in combinations of both conditions. Throughout of 13 chironomids species exposed to pesticides, there was no phylogenetic signal to enzymes responses, but to *Chironomus* species there was a trend to AChE and EST-α enzymes. Selection pressure showed the main driver of enzymatic responses due to the fact same species collected in different sites had no similar levels of activity. Chironomidae larvae have a remarkable physiological plasticity that allows them to survive in challenging environments, even mortality being not observed changes were detected demonstrating homeostasis imbalance. In the environment, even suffering with selection pressure, chrinomids are alive and resistants, this status can reflect in some fitness cost of this species in a long-term.

Keywords: Chironomidae, sediment, biochemical biomarkers, gene expression, comparative biology

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PRÓLOGO

A sociedade humana ao longo da sua evolução conquistou grandes avanços na área do conhecimento, este acúmulo de informação nas áreas da ciências exatas e da terra, engenharias e biológicas propiciou o desenvolvimento de tecnologias que permitiram notavelmente a melhora da qualidade de vida da população (ROCO; BAINBRIDGE, 2003).

A Revolução industrial, no século XVIII na Inglaterra, foi um marco inicial para a ascensão do setor produtivo, tornando os produtos mais acessíveis, estimulando o consumo e consequentemente ampliando os lucros. Todas estas características promoveram o êxodo rural, ocasionando um aumento populacional acelerado dos centros urbanos, e elevasse a demanda por alimentos e produtos manufaturados (ARAÚJO; PIMENTEL, 2015).

Por um lado, a busca por avanços e melhoria da qualidade de vida da população é imprescindível e deve ser sempre incentivado, em contrapartida a revolução industrial e tempos precedentes à revolução estão relacionados a liberação de resíduos no meio ambiente (UGLIETTI et al., 2015). Antes da revolução os resíduos liberados eram basicamente compostos por matéria orgânica, os quais, apesar dos impactos, não geravam grandes prejuízos. Porém, com a instalação de fábricas e produção em larga escala, novos produtos químicos foram inseridos no mercado, aumentando a diversidade dos resíduos liberados na natureza. Portanto, melhorias para o desenvolvimento da sociedade humana devem ser constantemente incentivadas e buscadas, porém a questão chave para os tempos modernos é que se utilizem estratégias sustentáveis para a diminuição do impacto nos ecossistemas (GIDDINGS; HOPWOOD; O'BRIEN, 2002).

Enquanto a preocupação com alternativas sustentáveis é um desafio para governos atuais e futuros, a questão da poluição química ainda é um problema nos dias de hoje.

O ecossistema aquático geralmente é o destino final da contaminação provinda das atividades antrópicas. O compartimento sedimento representa um depósito dessas substâncias, porém existe uma dinâmica de troca entre sedimento e coluna d'água que pode ressuspender esses xenobióticos (EGGEN et al., 2004; PÉRY et al., 2008).

Além do desafio por estratégias sustentáveis, o maior desafio é o encontrado pelos organismos que vivem nesses ambientes, pois eles são constantemente expostos à misturas complexas de poluentes (DIAMOND et al., 2015).

Tanto estudos avaliando a toxicidade de contaminantes isolados como estudos verificando a toxicidade de amostras do campo são necessários para diferentes finalidades em um contexto ecotoxicológico. Em avaliações de xenobióticos isolados, é possível controlar as variáveis em laboratórios e detectar uma relação de causa e um efeito. Porém a informação provinda deste tipo de estudo se afasta em grande maioria das condições reais encontradas no meio ambiente, mas essa resposta é extremamente importante para questões de legislação e regulação ambiental (BARBERA; MCCONNELL, 1990; REVESZ, 1999).

Já nos estudos que utilizam amostras de campo dificilmente é possível realizar a associação de causa e efeito devido a complexidade das interações existentes. Mas essas respostas estão mais próximas do que acontece na natureza, além de serem utilizadas tanto como questão de avaliação de risco ecológico de áreas mas também elucidação de efeitos reais no campo (SEGNER, 2007). O cenário ideal é que seja sempre realizado os dois tipos de estudos na tentativa de compreender como os contaminantes interagem com a biota.

Levando em consideração a contaminação do ambiente aquático e sedimento, existe uma fauna dependente do sedimento, que o usa para proteção, desenvolvimento e forrageamento. Essa comunidade é formada principalmente por invertebrados sendo a maioria insetos (MERRITT; CUMMINS, 1984).

Dentre os insetos, os organismos da família Chironomidae (DIPTERA) são os mais abundantes e amplamente distribuídos, sendo que suas larvas utilizam o ambiente aquático em toda ontogenia, e são consideradas modelos para estudos ecotoxicológicos da qualidade de sedimentos límnicos (CAREW et al., 2007; TRIVINHO-STRIXINO, 2011).

As larvas de Chironomidae são consideradas por alguns autores como organismos tolerantes a metais e baixas concentrações de oxigênio devido a presença de hemoglobinas na sua hemolinfa (LEE et al., 2006; CAREW et al., 2007). Esses organismos apresentam um relevante papel ecológico na ciclagem de nutrientes além se serem o principal elo entre produtores e consumidores nas teias tróficas (BERG; HELLENTHAL, 1992). Mesmo sendo considerados tolerantes,

sobreviver em ambientes inóspitos possuem custos biológicos para esses organismos.

Uma forma de mensurar custos biológicos ou condições de estresse nos organismos é utilizando biomarcadores ou *endpoints* de contaminação ambiental. Os biomarcadores são respostas mensuráveis induzidas por contaminantes em sistemas biológicos (componentes celulares, bioquímicos, genéticos, processos fisiológicos) (BENCIC, 2015).

KRULL & BARROS (2012) realizaram uma revisão crítica sobre o cenário ecotoxicológico no Brasil, em que levantaram algumas questões chave, e duas delas foram destacadas na presente tese. A primeira é que os insetos não foram considerados na avaliação de tipos de organismos utilizados devido a baixa exploração desse grupo nos estudos, sendo peixes (29,2%) e cladóceros (22,1%) predominantes. A segunda foi que a toxicidade relacionada ao sedimento, seja sedimento-integral ou elutriato, é explorada em menos de 7% dos estudos enquanto exposição via água representa 83,4%.

Diante do cenário apresentado, a presente tese teve como incentivo o fato que as larvas de Chironomidae são amplamente distribuídas, importantes na ecologia funcional dos ecossistemas, e estão constantemente expostas a xenobióticos. Assim, buscou-se avaliar respostas fisiológicas de larvas de Chironomidae sob DESAFIOS da sobrevivência em degradação ambiental por meio de biomarcadores.

No capítulo 1: "Whole-sediment tests from different anthropogenic influence sites in *Chironomus santicaroli:* biochemical and histological effects", incentivados pelo cenário atual da ecotoxicologia no Brasil, buscou-se entender como sedimento coletado do campo, constituído por suas interações entre aspectos químicos e físicos, interfere na homeostase das larvas, avaliada pela utilização de biomarcadores bioquímicos e histológicos.

O capítulo 2: "Dissolved oxygen combined with arsenic exposure in *Chironomus dilutus* (Diptera: Chironomidae): implications to respiration and antioxidant responses", foi realizado no período do doutorado sanduíche na North Carolina State University, USA. Fomos incentivados pela constante liberação do arsênio presente no resíduos das usinas termoelétricas nos Estados Unidos combinados com a constante eutrofização dos corpos dágua. Assim, buscou-se entender como larvas de Chironomidae, tratados na literatura como tolerantes a

estas condições (metal + hipóxia), respondem à interação dos mesmos considerando-se a defesa antioxidante (expressão gênica da enzima GST) e respiração (consumo de oxigênio e expressão gênica de hemoglobinas) processo tão peculiar nesses organismos.

Por fim o capítulo 3: "Biochemical responses across Chironomidae (Diptera) species exposed to pesticides: do selection pressure or evolutionary history drive the tolerance?", incentivados pela constante afirmação que aspectos comparativos devem ser inseridos em estudos biológicos modernos. Assim, buscou-se investigar se as respostas bioquímicas em Chironomidae possuem um componente mais relacionado com a história evolutiva do grupo ou são um reflexo da história dos ambientes que elas compartilham.

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CAPÍTULO I

WHOLE-SEDIMENT TESTS FROM DIFFERENT ANTHROPOGENIC INFLUENCE SITES IN *Chironomus santicaroli* (DIPTERA: CHIRONOMIDAE): BIOCHEMICAL AND HISTOLOGICAL RESPONSES*

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Abstract: In aquatic ecosystems, sediments are considered the deposit of chemical contaminants. Dwelling organisms thrive in this compartment being exposed to a complex mixture of xenobiotics. The aim of this study was to evaluate biochemical and histological responses in *Chironomus sancticaroli* exposed to contaminated sediments. We exposed the chironomid tube-dwelling species to whole sediments from five rivers and five reservoirs under different anthropogenic activities (conservation, industrial and farming) from São Paulo State, Brazil and to a control group. It were measured the following biochemical biomarkers: AChE (acetylcholinesterase), EST-α and EST-β (carboxylesterases alpha and beta), GST (glutathione *S*-transferase), SOD (superoxide dismutase), CAT (catalase) and the stress oxidative biomarker LPO (lipid peroxidation) beyond histological biomarker. The chemical analysis showed presence of metals, polycyclic aromatic hydrocarbons, organochlorine pesticides and polychlorinated biphenyls. All treatments had promoted alterations in biochemical biomarkers in some cases in opposite of usually observed behaviors, e.g. increase of AChE activity and decrease of SOD and LPO, demonstrating the complexity of the effects of mixtures. In treatments from Industrial and Farming, the larvae had an increase of lipid peroxidation, showing a stress oxidative status. Fat body was the most sensitive organ exhibiting alteration in almost all treatments. Even reference sites weas found the presence of contaminants, determining physiological alteration in the larvae. These results showed that chironimid larvae exposed to contaminated sediment were in stress condition. In the environment, these larvae must to produce more enzymes to thrive, and in a long term have metabolic disturbances.

1 Introduction

One of the big challenges of the ecotoxicological studies is understanding the adverse effects of complex mixtures to organisms (Eggen et al., 2004). The major sources of aquatic contamination are the industrial and agricultural effluents/residues (Ebenstein, 2012; Ross and Rupe, 2015). Recently, there was an increase of research evaluating the combination of chemical substances in laboratory and field experiments (Bueno-Krawczyk et al., 2015; Schoenfuss et al., 2016).

In artificial complex mixtures is possible to control combinations of pollutants to isolate variables and estimate some causes and effects. However, there is a

variety of chemical contaminants and environmental conditions that would influence the biological responses being tough to carry out experiments for all substances (Beyer et al., 2014).

Field samples are the most complex mixture; the difficult task is to identify and quantify the substances and to address some effects to the specific chemical causer considering that multiple stressors can behave additive/synergistically or antagonistically (Piggott et al., 2012). Nevertheless, to evaluate toxicity using natural samples can give results closer to real scenarios of exposure. Therefore, this information is valuable to environmental risk assessment, mainly from the organisms's point of view (Beyer et al., 2014).

The aquatic ecosystem comprises the water, sediment, and living organisms and ecotoxicological studies using field samples can evaluate water and sediment toxicity (Tavakoly-Sany et al., 2014). During the last few years, research only considering water column analysis was not enough to water assessment criteria (EPA, 2000). Some pollutants of aquatic systems have affinity for particulate material and can be associated with the sediment (Traunspurger and Drews, 1996). The sediments are considered the final destination of contaminants being a storage place and the source of these substances when they are resuspended (Roberts, 2012).

Studies using sediments became very common, but this compartment is still less explored than water-column. Initially, the aqueous phase of sediment was tested in water-column organisms (e.g. *Daphnia* sp.) (Diepens et al., 2014). But there was a need to evaluate the adverse effects in benthic organisms using whole-sediments tests because that is a more realistic condition (Liß and Ahlf, 1997).

A list of freshwater species to be used in whole-sediment tests was provided by Traunspurger and Drews (1996) and in addition to other species, it was highly recommended the use of *Chironomus* genus due to its behavior in the sediments and feeding habits; high ecological relevance; sensitivity and the large database of its responses to various toxicants (Traunspurger and Drews, 1996). Chironomids are tube-dwelling organisms and their larval stage is closely related to the sediments that play a protection role to these animals (Halpern et al., 2002).

Despite these organisms being considered tolerant to some substances (e.g. some metals) (Carew et al., 2007), the biological costs of living in contaminated environments can be measured using biomarkers (Van Gestel and Van Brummelen, 1996; Cortes et al., 2016).

In several chironomids species were evaluated the adverse effects of different substances in single or mixtures experimental designs; in laboratory contamination or samples from the field. Furthermore, was applied endpoints such as survival, growth, reproduction and morphological deformities and more recently biochemical and molecular biomarkers (Fisher et al., 2003; Choi, 2004; Arambourou et al., Printes et al., 2011; Cao et al., 2012; Choi, 2014;Ozáez et al., 2016).

Although there are several studies showing effects on chironomid fauna, whole-sediments tests applying biomarkers were less explored (only three papers). Regarding biochemical biomarkers studies, they were restricted to AChE (Acethylcholinesterase), GST (Glutathione S-transferase), EROD (7-Ethoxyresorufin-O-deethylase) and MT (Metallothionein) (Olsen et al., 2001; Printes et al., 2011; Duran et al., 2012). As far as we know, there is no papers demonstrating histological effects in chironomids exposed to chemical substances (Richardi et al., 2015). Multi-levels analysis provides an integrated response to the mixture of xenobiotics and favors the detection of risk assessments.

For this study the freshwater benthic larvae used as model were *Chironomus sancticaroli* (Diptera: Chironomidae). This species was considered synonymy to *Chironomus xanthus*, Rempel, 1939 (Trivinho-Strixino, 2011), being easily reared in the laboratory (Fonseca and Rocha, 2004). It was used as ecotoxicological model in studies in Brazil (Moreira- Santos et al., 2005; Sotero-Santos et al., 2007; Printes et al., 2011).

Understanding if chemical contaminants are bioavailable in sediments from field samples is necessary to environmental risk assessment. To shed light to this question is recommended to perform an integrated approach using native species. Thus, biochemical biomarkers and histological analysis were applied in whole-sediments tests in this study to evaluate the toxicity of sediment samples.

This study aimed to evaluate the biochemical and histological responses of *Chironomus sancticaroli* to chemical contamination, and ecological integrity of rivers and reservoir sediments from farming, industrial and reference areas during winter and spring through physical and chemical analysis and chronic toxicity tests.

2 Material and Methods

2.1 Test animals

Larvae of *Chironomus sancticaroli* were obtained from a laboratory culture of Laboratório de Entomologia Médica e Veterinária at Universidade Federal do Paraná. They were reared under standard laboratory conditions following toxicity tests guidelines (OECD, 2004a, 2004b). Egg masses were transferred to trays (2 L) containing reconstituted water with 1.2 $mg.L^{-1}$ hydrated $CaSO_4$, 0.08 $mg.L^{-1}$ KCl, 2.44 $mg.L^{-1}$ $MgSO_4x7H_2O$, and 1.92 $mg.L^{-1}$ Na_2CO_3, conductivity of 160 µS cm^{-1}, pH 7.2 and hardness 16 mg L^{-1}. Larvae were fed TetraMin® fish food, as substrate was used quartz sand 50-70 mesh (Sigma-Aldrich®). Cultures were maintained under constant aeration at 25 °C and standard light–dark periods 12:12h. Experiments were carried out using exclusively first instar larvae, and the larval stage was determined based on the size of head capsule (Richardi et al., 2013).

2.2 Study areas and sediment sampling

São Paulo State Environmental Agency - Brazil (CETESB) established a network monitoring of water and sediments quality. The State was divided in Units of Water Resources Management considering the different antrophic influence (e.g. industrial, farming or conservation).

For this study were selected ten sites (five rivers and five reservoirs) from this network monitoring (Figure 1). In the winter/spring (dry season) of 2013, the sediment samples were collected from the depositional edge of the rivers and profundal zone of the reservoirs using a van Veen Grab Sampler by CETESB. Samples were taken in triplicate, and for the reservoirs were considered only the first 6 cm of sediment column. The samples were stored at -4°C up to a month, before bioassays they were mixed manually.

Reference sites:

The Claro-Guaçu River (RC) (23°41’49”S, 45°28’58”W – Caraguatatuba, SP) is used to water capitation for public supply.

Chavantes reservoir (PAXA) (23°09’46”S, 49°41’28”W – Timburi, SP) was built to maintain an electric plant. It has influence of intensive sugar cane and grain production (corn, soil, wheat) (Jorcin and Nogueira, 2005).

Farming influence sites*:*

Ilha Solteira reservoir (IS) (20°20’44”S, 51°20’31”W – Ilha Solteira, SP) has a regime of hydraulic accumulation and, contribution to the flow of the Paraná River. In addition has a strong influence of aquaculture activities (Mallasen et al., 2012).

Santo Anastácio River (STA) (22°09’02”S, 52°09’55”W – Presidente Epitácio, SP) this site has a strong influence of the release of domestic loads from cities and slaughtering activities of cattle, dairy products and tanning activities (Leal et al., 2016).

In industrialization process site:

Peixe River (PEXE) (22°23’54”S, 46°50’48”W – Itapira, SP), this site has an influence of the release of domestic loads.

Industrial influence sites*:*

The Billings reservoir (BL) (23°47’11”S, 46°38’49”W – São Paulo, SP), the largest water body in the Metropolitan São Paulo region. Originally, it was built to produce hydroelectric power. It receive a great amount of contaminated water from Tietê and Pinheiros River (Soares and Mozeto, 2006).

Guarapiranga reservoir (GP) (23°41’22”S, 46°42’35”W – São Paulo, SP) is located in the metropolitan area of Sao Paulo city, and is an important drinking water reservoir for the city, providing 25% of its water needs (Mozeto et al., 2001).

Jaguari reservoir (JAG) (23°17’15”S, 46°13’10”W, - Santa Isabel, SP), its main goal is allow the flow from Paraíba do Sul River that provides water supply to cities of São Paulo, Rio de Janeiro states. It is located in a most developed industrial area in São Paulo, Rio de Janeiro and Minas Gerais state (Soares et al., 2016).

Piracicaba River (PCB) (22°41’57”S, 47°38’12”W – Piracicaba, SP) is located in one of the most industrialized regions of São Paulo State, it receives effluents from industries, domestic waste and other human activities. In addition to its localization is in one of the largest sugar cane producing regions in the world (Botelho et al., 2013). It is a place to water captation for public supply.

Claro River (LC) (22°24’44”S, 47°32’40”W – Rio Claro, SP) is located in a urban region in the Rio Claro city and receives effluents from this city

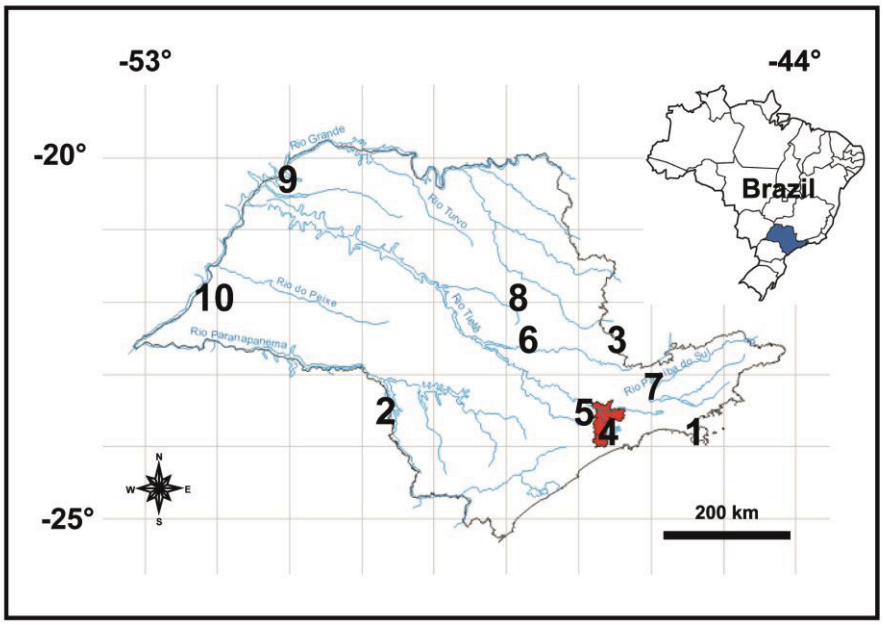

Figure 1: Sampling sites from São Paulo State. Conservation (1: Claro-Guaçu River, 2: Chavantes Reservoir, In industrialization process (3: Peixe River), Industrial (4: Billings Reservoir, 5: Guarapiranga Reservoir, 6: Piracicaba River, 7: Jaguari Reservoir, 8: Claro River), Farming (9: Ilha Solteira Reservoir, 10: Santo Anastácio River). Blue region in the Brazil map is São Paulo State. Red region in the São Paulo State map is São Paulo city metropolitan region.

2.3 Exposure conditions

For experimental treatments, 100 first instar larvae were exposed for 8 days (time to larvae reaching the fourth instar) in small glass aquaria (10 cm x 20 cm x 10 cm) containing 450 g of sediment and 750 ml of reconstituted water with 1.2 $mg.L^{-1}$ hydrated $CaSO_4$, 0.08 mg L^{-1} KCl, 2.44 $mg.L^{-1}$ $MgSO_4x7H_2O$, and 1.92 $mg.L^{-1}$ Na_2CO_3. Each treatment consisted of six replicates, for the control group was used quartz sand (Sigma-Aldrich®). The ratio of the depth of the sediment layer to the depth of the overlying water was 1:4, and the sediment surface provided per larvae was 2 cm^2 as suggested by OECD (2004a). The experiments were carried out under constant aeration at 25 °C and standard light–dark periods 12:12h. Each 48 hours, 2/3 of solution were renewed and added 5 ml of TetraMin® fish food aqueous solution (0.02 g/ml), and were measured pH, temperature, conductivity, total solids.

2.4 Analytical analysis of sediments

The analysis of sediments was performed at São Paulo State Environmental Agency (CETESB). The Table 1 contains which chemicals were analyzed, and the adopted methodology.

Table1: Chemicals analyzed and analytical methodologies adopted.

Chemicals	Analytical Method and Bibliography
Al, Pb, Cu, Cr, Fe, Mn, Ni, P, Zn (total) (mg/kg)	**Inductively coupled plasma optical emission spectrometry (ICP OES) (USEPA 6010C)**
As total (mg/kg)	**Inductively coupled plasma optical emission spectrometry (ICP OES), after acid digestion (USEPA 3051A)**
Hg total (mg/kg)	**Cold Vapor Generation Atomic Absorption Spectrometry, after acid digestion (USEPA 3051A)**
Total organic carbon (%)	**High-temperature catalytic oxidation (HTCO) with non-dispersive infrared (NDIR) detection (APHA 5310-B)**
Total Kjeldahl nitrogen (mg/kg)	**Ion chromatography (IC), after acid digestion. (APHA 4500 Norg)**
Acenaphthene (µg/kg)	**Liquid chromatography–fluorescence (Method 8310 - USEPA-SW 846)**
Anthracene (µg/kg)	
Benzo(a)anthracene (µg/kg)	
Benzo(a)pyrene (µg/kg)	
Benzo(b)fluoranthrene (µg/kg)	
Benzo(g,h,i)perylene (µg/kg)	
Benzo(k)fluoranthrene (µg/kg)	
Chrysene (µg/kg)	
Dibenzo(a,h)anthracene (µg/kg)	
Phenanthrene (µg/kg)	
Fluoranthrene (µg/kg)	
Fluorene (µg/kg)	
Indene(1,2,3-CD)Pyrene (µg/kg)	
Naphthalene (µg/kg)	
Pyrene (µg/kg)	
Aldrin((µg/kg)	**Gas chromatography-electron capture detection- CG/ECD (USEPA 8081B)**
Alpha BHC (µg/kg)	
Beta BHC (µg/kg)	
Delta BHC (µg/kg)	
Cis-chlordane (µg/kg)	
Trans-chlordane (µg/kg)	
DDD (µg/kg)	
DDE (µg/kg)	
DDT (µg/kg)	
Dieldrin (µg/kg)	
Endosulfan I (µg/kg)	
Endosulfan II (µg/kg)	
Endosulfan sulphate (µg/kg)	

Endrin (µg/kg)	
Heptachlor (µg/kg)	
Heptachlor epoxide (µg/kg)	
Hexachlorobenzene (µg/kg)	
Lindane (µg/kg)	
Methoxychlor (µg/kg)	
Mirex (µg/kg)	
Toxaphene (µg/kg)	
PCBs (mg/kg) Polychlorinated Biphenyls	**Gas chromatography-electron capture detection- CG/ECD (Method USEPA 8082A)**
Congenere 101 (µg/kg)	
Congenere 118 (µg/kg)	
Congenere 138 (µg/kg)	
Congenere 153 (µg/kg)	
Congenere 180 (µg/kg)	
Congenere 28 (µg/kg)	
Congenere 52 (µg/kg)	
Granulometry	**Determination of the granulometric distribution (Technical norm CETESB L6.160)**
Fixed solids, total solids, volatile solids, humidity	**Gravimetry (Method APHA 2540 G)**

2.5 Biochemical biomarkers

Each sample represents a pool with 2 whole larvae (final 4th instar). For each treatment was measured the enzymatic activity of 15 pools to seven biochemical biomarkers. The pool was homogenized in 1000 µl of Milli-Q water, and centrifuged at 12000 x g for 1 minute at 4°C. The supernatant was aliquoted in tubes/plates in a specific volume for each biomarkers and storage at -80°C freezer.

Each enzymatic assay was performed in triplicate, except catalase that was performed in four replicates, and measured at SpectraMax 190 Absorbance Microplate Reader (Molecular Devices®).

Acetylcholinesterase activity (AChE) was measured spectrophotometrically at 405 nm, following Ellman et al., 1961, modified to microplate by Silva de Assis (1998). The assay was carried out using 25 µl of sample, 200 µl of DTNB 0.75 mM, 50 µl of ATC 10 mM. It was incubated for 30 minutes and the kinetic was read each one minute for five minutes.

Alpha and beta esterases (EST-α, EST-β) were measured according to Valle et al., 2006 at 570 nm. It was used 10 µl of sample and was added 200 µl of alpha/or beta-naphthil acetate/Na phosphate 0.3 mM. The assay was incubated for 15

minutes, and then 50 µl of Fast Blue dye 0.3% was added and five minutes of incubation again.

Glutathione S-transferase was measured at 340 nm following Keen et al. (1976). To develop the assay 15 µl of sample, 195 µl of CDNB (21 mM) and GSH (10mM) solution were used and the kinetic was read each one minute for 20 minutes.

Catalase (CAT) activity was measured at 240 nm based on Aebi, (1984). It was used 5 µl of sample, 295 µl of reaction solution 80 mM (Tris base buffer 1M/ EDTA 5mM pH 8.0; hydrogen peroxide 30% and Milli-Q water. The kinetic was read each 15 seconds for five minutes

Superoxide dismutase (SOD) activity was measured at 440 nm according to Gao et al. (1998). In microtubes, were added 40 µl of samples and 885 µl of buffer (Tris base buffer 1M/ EDTA 5mM pH 8.0). After mixing, 50 µl of pyrogallol were added and incubated for 30 minutes. The reaction was interrupted, adding 25 µl of HCl 1 N. For each sample a control was carried out with the same procedures except the incubation.

Lipoperoxidation analysis (LPO) was carried out using the ferrous oxidation xylenol assay and measured at 570 nm (Jiang et al., 1992). The samples were diluted in methanol (1:1 v/v), centrifuged at 5000 x g for five minutes at 4°C. Supernatant (100 µl) plus 900 µl of Fox solution (orange xylenol 0.1 mM, BHT 4 mM, ferrous ammonium sulfate 2.5 mM and sulfuric acid 25 mM diluted in methanol). The assay was incubated for 30 minutes (dark).

The protein concentration in homogenates was carried out following Bradford's method (Bradford, 1976), using bovine serum albumin as standard measures at 620 nm. The assay was developed using 10 µl of sample and 250 µl of Bradford'reactive diluted in MIlli-Q water (1:5)

2.6 Histological biomarkers

Larvae were fixed in Duboscq solution for insects (Barth, 1958), for 4 h in an incubator at 56°C. After fixation, larvae were washed in 70% ethanol to fixative solution be removed; dehydrated in alcoholic series; diaphanization in xylene and infiltration in paraffin. 5 µm sections were stained using Hematoxylin–Eosin.

2.7 Statistical analysis

For enzymatic activities, the residual data were evaluated to the distribution by the Shapiro–Wilk test and the homocedasticity by Bartlett test, the comparison was performed using Unpaired Student t-test just among control group and treatment (river or reservoir sediment sample). In samples that the parametric tests presumptions did not agree was used Mann-Whitney test to compare the samples.

3 Results

3.1 Sediment chemical and physical analysis

All treatments had the presence of metals in some cases above TEL (Threshold Effects Level) and above PEL (Probable Effects Level) for Cu, Cr, Hg and Ni. At RC (Conservation), PEXE (In industrialization) and JAG (Industrial) were observed only metals. Polycyclic Aromatic Hydrocarbons (PAHs) were present in sites with some industrial influence (PEXE, BL, GP, PCB, CL) and STA (Farming). For the RC and PAXA (Conservation), JAG (Industrial) and IS (Farming) treatments were observed very low concentrations or not detected PAHs. Organochlorine pesticides (OCs) were observed at all different anthropic influence sites: PAXA (Conservation), BL and GP (Industrial) and IS and STA (Farming). OCs was not observed at RC (Conservation), PEXE (In industrialization), PCB, JAG and CL (Industrial) treatments. DDE pesticide was observed above PEL at BL (Industrial) and IS and STA (Farming) treatments. Polychlorinated Biphenyls (PCBs) were present only at BL (Industrial) treatment. The chemical concentrations and physico-chemical characteristics of sediments are shown in Table 2.

3.2 Biochemical Biomarkers

For all treatments, we observed alterations in biochemical responses. Regarding the conservation sites, Claro-Guaçu River (RC) and Chavantes Reservoir (PAXA) had a significant increase in AChE activity ($p<0.0001$ (RC) and $p=0.016$ (PAXA)) (Figure 2a). In addition, both EST-α ($p<0.0001$ (RC) and $p= 0.009$ (PAXA)) (Figure 2b) and EST-β ($p= 0.019$ (RC) and $p= 0.004$ (PAXA)) (Figure 2c) increased

their activity. The Claro-Guaçu River (RC) had a decrease in SOD activity ($p< 0.0001$) (Figure 2f) and GST activity decreases at Chavantes Reservoir samples ($p< 0.0001$) (Figure 2d).

About in industrialization process site, the Peixe River (PEXE) had just a decrease of SOD activity ($p<0.0025$) (Figure 2f) and an increase of lipid peroxidation ($p<0.0085$) (Figure 2g).

For the industrial influence sites, Billings Reservoir (BL) had a decrease of SOD activity ($p= 0.0046$) (Figure 2f) and lipid peroxidation ($p= 0.0017$) (Figure 2g). Guarapiranga Reservoir (GP) showed an increase of AChE activity ($p= 0.0049$) (Figure 2a), and decrease of GST ($p< 0.0001$) (Figure 2d) and SOD activity ($p= 0.0007$) (Figure 2f). At Piracicaba River (PCB) were observed an increase of EST-α ($p= 0.0019$) (Figure 2b) and EST-β ($p= 0.0001$) (Figure 2c) activity and decrease of GST ($p= 0.0013$) (Figure 2d) and SOD activity ($p= 0.0019$) (Figure 2f). Jaguari Reservoir (JAG) samples decrease the GST activity ($p< 0.0001$) (Figure 2d) and it was the only treatment that changed significantly CAT activity (decrease) ($p= 0.0019$) (Figure 2e). The Claro River showed an increase of AChE ($p= 0.0017$) (Figure 2a), EST-α ($p< 0.0001$) (Figure 2b) and EST-β activity ($p< 0.0001$) (Figure 2c) and a decrease of GST ($p< 0.0001$) (Figure 2d) and SOD activity ($p< 0.0001$) (Figure 2f).

In the farming influence sites we observed that Ilha Solteira Reservoir (IS) showed an increase of AChE ($p= 0.0041$) (Figure 1a) and EST-α ($p< 0.0001$) (Figure 2b) activity and a decrease of SOD activity ($p= 0.0019$) (Figure 2f) and lipid peroxidation ($p= 0.0019$) (Figure 2g). Santo Anastacio River (STA) had an increase of AChE ($p= 0.005$ – non parametric test) (Figure 2a) and EST-α ($p< 0.0001$) (Figure 2b) and EST-β ($p= 0.0007$) (Figure 2c) activity, and decrease GST ($p= 0.0030$) (Figure 2d) and SOD ($p= 0.04$) (Figure 2f), an increase trend of CAT ($p= 0.16$) not significant (Figure 2e) activity and a increase of lipid peroxidation ($p= 0.0007$) (Figure 2g).

3.3 Histological biomarkers

For all treatments except BL and JAG (Industrial) were observed vacuolization of trophocytes cytoplasm (visceral fat body) (Figure 3a-b). Only at PCB treatment (Industrial) was observed in all larvae a decrease of trophocytes cell volume (Figure

3c-d). For one larva in IS treatment (Farming) the oenocytes (parietal fat body) showed cytoplasm vacuolization (Figure 3e-f). A specific type of hemocyte, called plasmatocyte had cell proliferation in the hemolymph around the midgut beginning at RC (Conservation) and JAG (Industrial) treatments (Figure 3g-h). At RC (Conservation) and GP (Industrial) treatments showed an alteration in the midgut region I cells morphology (Figure 3i-j). Table 3 summarizes the frequency of histological alterations throughout the treatments.

Table 2: Results of chemical and physical characteristics of sediments utilized in toxicity tests. The reference values are according Canadian Council of Ministers of Environment (2001). Orange values: above TEL (Threshold Effect Level) and grey values: above PEL (Probable Effect Level).

Variable description		Reference values		RC	PAXA	PEXE	BL	GP	PCB	JAG	CL	IS	STA
Anthropic influence				Conservation		In industrialization	Industrial					Farming	
		TEL	PEL										
Metals and metalloids	Total Al (mg/kg)	-	-	61170	96042	95093	86984	75758	66079	77896	7752	87846	74876
	Total As (mg/kg)	5.9	17	2.22	10.1	1	8.38	10.8	1	1	1	6.09	6.8
	Total Cd (mg/kg)	0.6	3.5	1.05	0.69	0.5	1.68	0.92	0.76	0.5	0.5	0.5	0.55
	Total Pb (mg/kg)	35	91.3	12.6	24.6	43.5	53.2	62.8	23.7	42.4	15.5	19	23.6
	Total Cu (mg/kg)	35.7	197	20.3	55.3	22.1	98.9	3991	106	25.5	20.6	94.9	41.7
	Total Cr (mg/kg)	37.3	90	58.2	33.2	62.8	216	64.4	68.4	53.1	12.5	137	152
	Total Fe (mg/kg)	-	-	49770	59208	54966	85618	71869	52490	57371	13431	95134	49748
	Total Mn (mg/kg)	-	-	378	2129	708	496	1333	609	239	95.5	1043	639
	Total Hg (mg/kg)	0.17	0.486	0.05	0.13	0.07	0.878	0.143	0.12	0.11	0.04	0.11	0.1
	Total Ni (mg/kg)	18	35.9	28.7	18	25	47.9	27.5	22.1	18.4	10.3	39.7	41.4
	Total Zn (mg/kg)	123	315	95	93.4	83.1	238	160	201	66	49.3	74.6	61.7
Nutrients and Carbon	Total organic carbon (%)			1.11	1.7	1.66	3.59	4.87	2.31	1.79	1.39	1.81	1.57
	Total Kjeldahl nitrogen (mg/kg)			694	2133	1249	7309	4565	3279	2048	637	2337	1384
	Total P (mg/kg)	-	-	369	1152	1267	2462	3522	2037	1855	319	1440	1123
Polycyclic Aromatic Hydrocarbons (PAH)	Acenaphthene (µg/kg)	6.71	88.9	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
	Anthracene (µg/kg)	46.9	245	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20	< 20
	Benzo(a)anthracene (µg/kg)	31.7	385	< 20	< 20	< 20	< 20	< 20	< 20	< 20	33.4	< 20	< 20
	Benzo(a)pyrene (µg/kg)	31.9	782	10	10	14.3	32.5	17.2	29	10	66	10	10
	Benzo(b)fluoranthrene (µg/kg)	-	-	< 20	< 20	< 20	36.3	< 20	< 20	< 20	< 20	< 20	PI
	Benzo(g.h.i)perylene (µg/kg)	-	-	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80
	Benzo(k)fluoranthrene (µg/kg)	-	-	< 10	< 10	< 10	13.5	< 10	11.4	< 10	25.1	< 10	< 10
	Chrysene (µg/kg)	57.1	862	< 20	< 20	< 20	88.2	< 20	38.7	< 20	46.6	< 20	< 20
	Dibenzo(a.h)anthracene (µg/kg)	6.22	135	< 30	< 30	< 30	< 30	< 30	< 30	< 30	< 30	< 30	< 30
	Phenanthrene (µg/kg)	41.9	515	< 20	< 20	< 20	261	57.8	44.1	< 20	27.4	23.6	< 20
	Fluoranthrene (µg/kg)	111	2355	< 20	21.6	25.8	112	< 20	119	< 20	134	26.9	PI
	Fluorene (µg/kg)	21.2	144	< 20	< 20	< 20	54.7	< 20	< 20	< 20	< 20	< 20	< 20
	Indene(1.2.3-CD)Pyrene (µg/kg)	-	-	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80
	Naphthalene (µg/kg)	34.6	391	< 30	< 30	< 30	< 30	< 30	< 30	< 30	< 30	< 30	80.2
	Pyrene (µg/kg)	53	875	< 20	< 20	23	176	26.8	< 20	< 20	92.5	< 20	< 20
Organochlorine pesticides	Aldrin((µg/kg)	-	-	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
	Alpha BHC (µg/kg)	-	-	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Beta BHC (µg/kg)	-	-	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Delta BHC (µg/kg)	-	-	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Cis-chlordane (µg/kg)	-	-	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
	Trans-chlordane (µg/kg)	-	-	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
	DDD (µg/kg)	3.54	8.51	< 0.5	1.27	< 0.5	2.95	1.26	< 0.5	< 0.5	< 0.5	< 0.5	1.32
	DDE (µg/kg)	1.42	6.75	< 0.5	2.08	1.2	18.6	2.65	1.4	0.79	1.12	10.5	7.24
	DDT (µg/kg)	1.19	4.77	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	1.86
	Dieldrin (µg/kg)	2.85	6.67	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
	Endosulfan I (µg/kg)			< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
	Endosulfan II (µg/kg)			< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
	Endosulfan sulphate (µg/kg)			< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5
	Endrin (µg/kg)	2.67	62.4	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
	Heptachlor (µg/kg)	0.3	10,00	< 1.25	< 1.25	< 1.25	2.49	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25
	Heptachlor epoxide (µg/kg)	0.6	2.74	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25
	Hexachlorobenzene (µg/kg)			< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75	< 0.75
	Lindane (µg/kg)	0.94	1.38	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25	< 1.25
	Methoxychlor (µg/kg)			< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Mirex (µg/kg)	7	1300	< 0.5	< 0.5	< 0.5	< 0.5	1.65	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
	Toxaphene (µg/kg)			< 100	< 100	< 100	< 100	< 100	< 100	< 100	< 100	< 100	< 100
Polychlorinated Biphenyls	PCBs (mg/kg) Polychlorinated Biphenyls	34.1	277										
	Congenere 101 (µg/kg)			< 2.5	< 2.5	< 2.5	20	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Congenere 118 (µg/kg)			< 2.5	< 2.5	< 2.5	22.6	2.54	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Congenere 138 (µg/kg)			< 2.5	< 2.5	< 2.5	13.6	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Congenere 153 (µg/kg)			< 2.5	< 2.5	< 2.5	21.1	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Congenere 180 (µg/kg)			< 2.5	< 2.5	< 2.5	11.6	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Congenere 28 (µg/kg)			< 2.5	< 2.5	< 2.5	16.2	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
	Congenere 52 (µg/kg)			< 2.5	< 2.5	< 2.5	13.1	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5	< 2.5
Physico-chemicals	Moisture content (%)			56.2	80.4	60.4	87.3	85	63.8	67.1	39	81.5	67.6
	pH			6.67	7.28	6.95	6.41	6.38		7.15	6.93	7.06	7.12
	Redox Potential (mV)			-24.1	-148	-92.3	-115	-87.8		-184.3	23.1	-202	-127.1
	Fixed solids (%)			93	85	87	79	81	88	88	97	88	91
	Total solids (%)			52	21	38	11	15	38	32	63	19	34
	Volatile solids (%)			7	15	13	21	19	12	12	3	12	9
Granulometry	Coloration				Brown	Brown			Grey		Brown	Brown	Brown
	Sand (%)			77.59	3.44	13.11	6.97	7.39	5.41	20.47	83.12	1,00	12.34
	Clay (%)			6.7	79.14	49.66	69.92	63.19	47.74	63.17	10.51	93.35	80.32
	Silt (%)			15.71	17.42	37.23	23.11	29.42	46.85	16.36	6.37	5.65	7.34
	Classification			sand	sand	silty clay	silty clay	silty clay	silty clay	sandy clay	sand	clay	clay

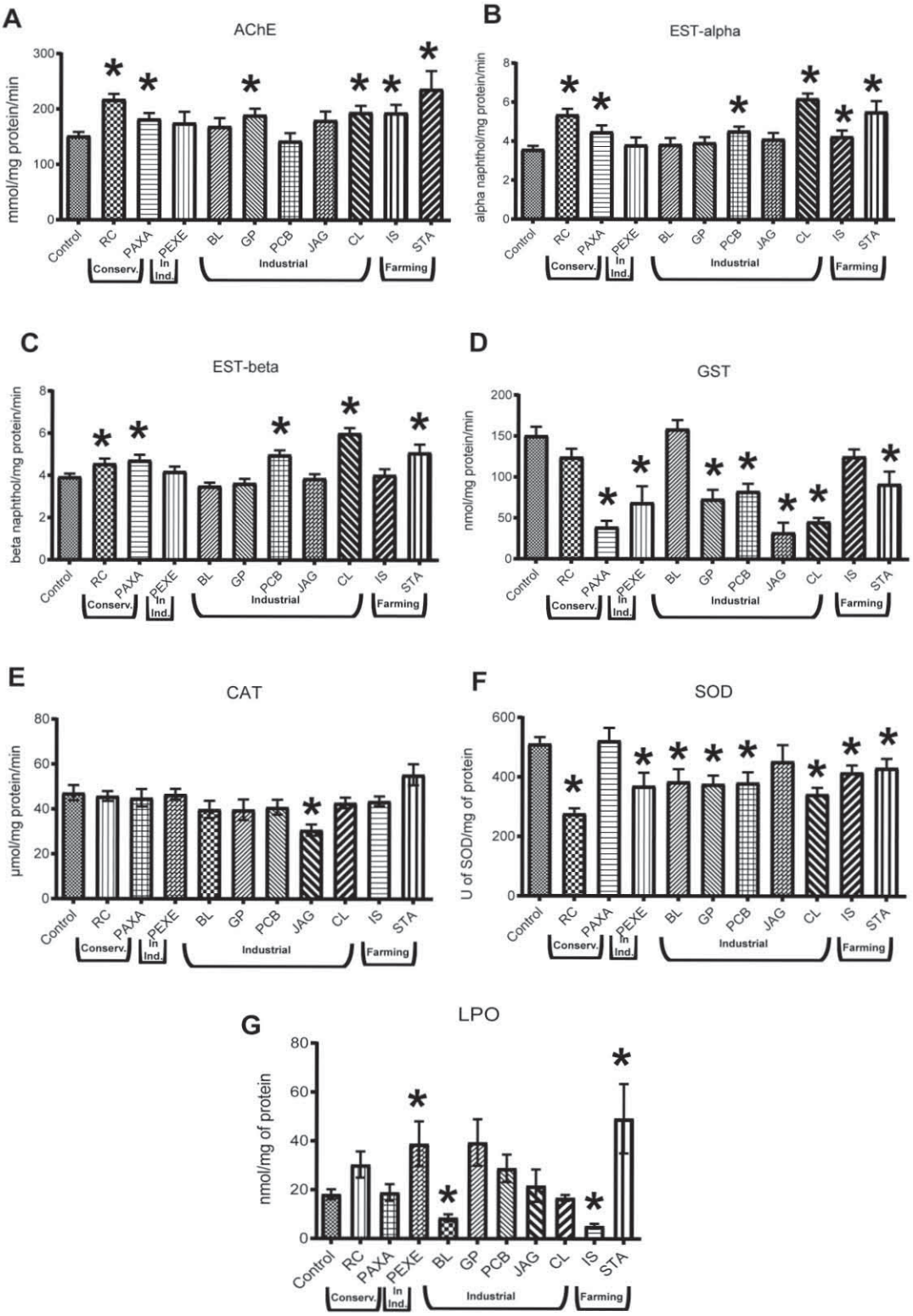

Figure 2: Biochemical biomarkers in *Chironomus sancticaroli* exposed to contaminated sediments. A: AChE, acetylcholinesterase, B: EST-α, esterase alpha, C: EST-β, esterase beta, D: GST, Glutathione *S*-transferase, E: CAT, catalase, F: SOD, superoxide dismutase, G: LPO, lipid peroxidation. RC: Claro-Guaçu River; PAXA: Chavantes reservoir; PEXE: Peixe River; BL: Billings reservoir; GP: Guarapiranga reservoir; PCB: Piracicaba River; JAG: Jaguari reservoir; CL: Claro River; IS: Ilha Solteira rerservoir and STA: Santo Anástácio River. Values are expressed as mean ± standard error of mean. Asterisks indicate statistically significant differences ($p < 0.05$) compared to control group; Student t-test (n=13-15).

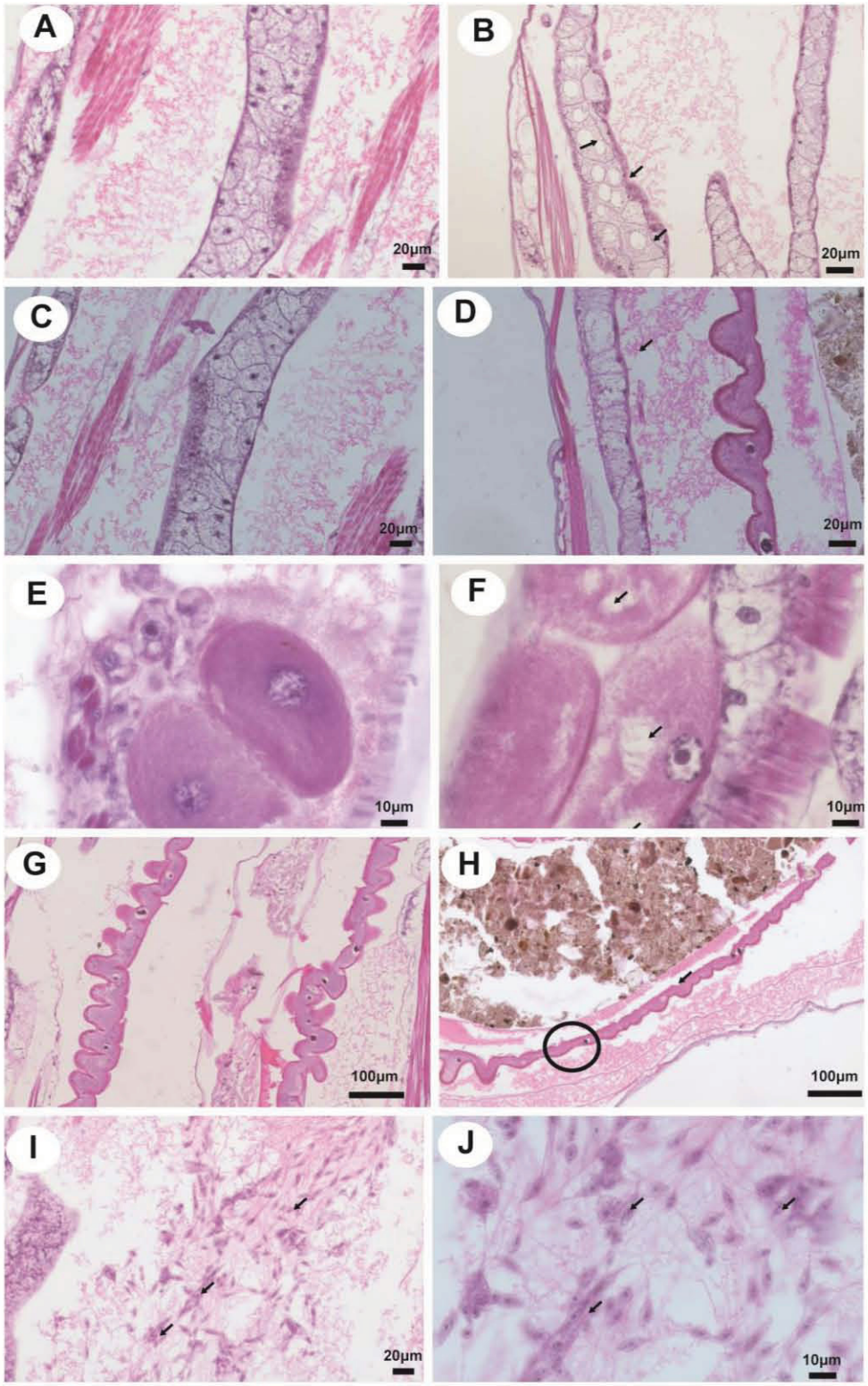

Figure 3: Histological alterations in *Chironomus sancticaroli* exposed to contaminated sediments. A and C: visceral fat body in the control group. B: vacuolization of fat body cytoplasm cells in the treatment, arrows are indicating vacuoles. D: Reduction of volume of fat body (arrow). E: Oenocytes in control group. F: Vacuolization of oenocytes, arrows are indicating vacuoles. G: Midgut (region I) in control group. H: Midgut in treatments, circle and arrow are indicating alteration in epithelium morphology. I and J: Appearing of hemocytes (plasmatocytes) in the hemolymph in treatments.

Table 3: Frequency of histological alterations in *Chironomus sancticaroli* submitted to treatments. The sample size for each treatments was five (n=5). Conservation (RC: Claro-Guaçu River; PAXA: Chavantes reservoir); In industrialization process (PEXE: Peixe River); Industrial (BL: Billings reservoir; GP: Guarapiranga reservoir; PCB: Piracicaba River; JAG: Jaguari reservoir; CL: Claro River); Farming (IS: Ilha Solteira rerservoir and STA: Santo Anástácio River).

Influence	Conservation		In Industrializ.	Industrial					Farming	
Treatment	RC	PAXA	PEXE	BL	GP	PCB	JAG	CL	IS	STA
Trophocyte vacuolization	4/5	2/5	1/5	0	5/5	5/5	0	3/5	4/5	2/5
Trophocyte volume reduction	0	0	0	0	0	5/5	0	0	0	0
Oenocytes vacuolization	0	0	0	0	0	0	0	0	1/5	0
Plasmatocytes proliferation	2/5	0	0	0	0	0	1/5	0	0	0
Midgut region I alteration	1/5	0	0	0	2/5	0	0	0	0	0

4 Discussion

Analyzing these responses by treatment we can observe that for all treatments larvae showed a stress framework in different levels. But if we look at the biochemical biomarkers there were no clear relationships between activity levels and sediment chemical and physical characteristics.

AChE activity was increased at RC and PAXA (Conservation), GP and CL (Industrial), IS and STA (Farming) treatments. Similar results were reported for *Chironomus riparius* in collected larvae from the field (Duran et al., 2012) and *in situ* exposure (Olsen et al., 2001), in both studies the authors only measured metals concentration. It is expected a decrease in its activity when organisms are exposed to pesticides resulting in the accumulation of acetylcholine neurotransmitter in the synaptic gap and disrupting of nervous system function (Domingues et al., 2010). In addition, exposure to some metals has unclear relationship with a decrease of AChE activity (Frasco et al., 2005).

All these treatments where we observed increase of AChE activity are contaminated with only metals or metals combined with HPAs/OPs. A species of marine fish *Seriola dumerilli* exposed to cadmium showed an increase of

acetylcholinesterase activity at the lowest concentration tested (50 µg/kg) (Jebali et al., 2006). In addition, *Danio rerio* exposed to copper (60 µg/kg) had an increase of AChE after 2 days of exposure (de Lima et al., 2013). Cadmium and copper were present in all these samples at much higher concentrations (up to 500 µg/kg). It is very difficult to identify the responsible for these enzymatic responses when we talking about complex mixtures. Beyond Cd we could find other metals, organic compounds and non-measured substances in these samples. Furthermore, metals exposure can mask AChE evaluation in different levels using Ellman et al. (1961) method due to interaction of metals and reagents (Frasco et al., 2005).

All these chemicals may interact with each other, resulting in synergism/or antagonism changing the expected toxicity and giving misinterpretations in the biochemical responses (Domingues et al., 2010). Pavlov (1996) studying AChE to Cd exposure in the freshwater fish *Rutilus rutilus* found an activity increase, this author associated the increase of this enzyme with stress framework that reflects changes in protein synthesis in animals that are adapting to new conditions.

The enzymes EST-α and EST-β had a consistent behavior, showing an increase of their activity at RC and PAXA (Conservation), PCB and CL (Industrial), STA (Farming). IS (Farming) treatment had an increase only in the EST-α activity. Carboxylesterases play a role of xenobiotics detoxification (Yu et al., 2009) by sequestrating and metabolizing exogenous substances (Cacciatore et al., 2013).

According to Terriere (1984), these enzymes can be induced by pesticides, but some authors found other chemicals inducing their activity, e.g. metals (Zhang et al., 2014), HPAs (Nousiainen et al., 1984). There is no clear relationship between the pollutants present in these samples and enzymes responses. But all of them had pesticides and/or metals and/or hydrocarbons. Our results show that larvae were expressing these enzymes in an attempt to eliminate the chemicals. Even though it is remarkable that treatments with similar chemical composition had not effect on these enzymes.

GST enzyme had a decrease in its activity at PAXA (Conservation), GP, PCB, JAG, CL (Industrial) and STA (Farming). This enzyme has a very low substrate specificity and can show responses to a wide range of xenobiotics (Lagadic et al., 1994). Some authors observed a GST activity enhancement in the contaminated conditions in different organisms from invertebrates to vertebrates (Guiloski et al., 2015; Oexle et al., 2016; Renzi et al., 2016). In contrast, a decrease also was

reported (Yu et al., 2015). For chironomids both behaviors can be observed, but it was more reported a decrease of GST activity by several authors (Rakotondravelo et al., 2006; Jin-Clark et al., 2008; Domingues et al., 2009; Printes et al., 2011; Wiseman et al., 2013; Rodrigues et al., 2015; Yu et al., 2015). However, it remains unclear the biological meaning of the use of GST as biomarker in chironomids and it had been questioned in other studies (Hirthe et al., 2001, Callaghan et al., 2002). GSTs are a multiple isoenzymes codified by a large family of genes, specifically 13 genes were identified for *Chironomus riparius* (Nair and Choi, 2011). The unclear behaviors of GST activity thoroughly different studies may be related to the expression of different isoenzymes with varying sensitivity for the xenobiotics (Olsen et al., 2001).

CAT enzyme had a decrease of its activity just at JAG treatment (Industrial) and no changes were observed for the other treatments. Catalase is an antioxidant enzyme that plays the hydrogen peroxide decomposition in oxygen and water (Lopez-Martinez et al., 2008). JAG treatment was contaminated basically with metals (being Pb, Cr, Ni above TEL (Threshold Effects Level)). Some studies showed a decrease of CAT activity caused by metals as well (Atli et al., 2006; Atli and Grosell, 2016), deltamethrin (Sayeed et al., 2003) and UV radiation (Oexle et al., 2016). Explanations about decrease of CAT activity give some hypothesis; maybe occurred an imbalance of ROS (reactive oxygen species) formation followed by antioxidant defense system dysfunction for enzyme activity enhancement or compensation for GSH (not measured in this study) depletion (Fahmy et al., 2014). An alternative hypothesis is related with the direct binding of metals to –SH groups on the enzyme molecule (Atli et al., 2006). In addition, no changes in CAT activity may be related to an increase of other antioxidant enzymes such as GPX or metallothioneins (Atli et al., 2006).

SOD enzyme had a decrease of its activity in all treatments except PAXA (Conservation) and JAG (Industrial). Superoxide dismutase is a metalloenzyme that catalyzes the dismutation of the superoxide radical into hydrogen peroxide and oxygen. Antioxidant enzymes can increase the activity when the organisma are exposed to contaminants (Park et al., 2012). Similar ours results some author found a decrease of SOD activity (Kristoff et al., 2008; An et al., 2009; Liu et al., 2010; Shao et al., 2012). According to Sun et al. (2007) the excess of O_2 radicals can influence negatively SOD activity or make them inactivated. Furthermore, at high

levels of contaminants as observed in these samples (metals mainly) is common to observe decrease of SOD activity (Lin et al., 2007).

When oxidative stress is observed, often CAT and SOD show an enhancement in their activities to scavenge ROS. However, these antioxidant enzymes are sensitive to damage by ROS (Goldstone et al., 2006). Probably high levels of ROS were produced that led to a continuous decrease of antioxidant levels (Kristoff et al., 2008). We did not observe a coordinated response, maybe because the oxidative attack may respond in different ways (Liu et al., 2010). Metallothioneins have an important role in detoxification of metals, but these proteins can also act as free radical scavengers. (Andrews, 2000; Sato and Bremner, 1993).

Lipid peroxidation had an increase at PEXE (In industrialization process) and STA (Farming) and a decrease at BL (Industrial) and IS (Farming). LPO is a damage caused by ROS and the main target is the cell membranes, causing its disruption or dysfunction allowing the DNA attack (Farmer and Mueller, 2013). When is observed an increase of lipid peroxidation the organisms are in an oxidative stress status (Valavanidis et al., 2006). PEXE and STA organisms also had a decrease of SOD activity; probably high levels of ROS caused damage in SOD enzyme and lipids of membrane. In the freshwater bivalve *Unio tumidus* exposed to sediments, LPO also was elevated when antioxidant defenses were depleted (Cossu et al., 2000).

Regarding histological alterations, more prominent alterations were observed in the visceral fat body throphocytes. The cytoplasm of fat body throphocytes was vacuolated in almost all treatments except BL and JAG (Industrial) and in only one larva at IS (Farming) had vacuolization of oenocyte cytoplasm. The diplopod *Rhinocricus padbergi* exposed to sewage sludge also showed fat body vacuolated (de Godoy and Fontanetti, 2010). Vacuolization of cells cytoplasm is an indicative of necrosis process initiation and consequently cell death (Edinger and Thompson, 2004). In addition, only at PCB (Industrial) treatment we observed decrease of trophocyte volume. This treatment was the only one where mortality was observed (CETESB, 2013). This biomarker had a direct relationship with mortality in this study and may have a remarkable use in future investigations.

Midgut (Region I) showed cell morphology alteration at RC (Conservation) one larva and GP (Industrial) two larvae. This alteration also was observed in the ant *Atta sexdens rubropilosa* exposed to boric acid, and maybe can affect the organisms feeding (Sumida et al., 2010). The increase of plasmatocytes often is related to

pathogens, their function is to perform phagocytosis (Borowska and Pyza, 2011), maybe these treatments made the organisms susceptible to pathogens infection.

It is clear we did not observe relationship between sample that had similar chemical compositions and their biomarkers responses. For example, BL was the most contaminated site, but we observed few biochemical and no histological responses. PCB treatment does not have a prominent difference in chemical composition, but was the only one that showed mortality (CETESB, 2013). RC, PEXE and JAG were contaminated basically with the same metals, but we did detect consistent biochemical responses.

Some hypothesis can be proposed to explain our results; metals were present in all treatments. The toxicity and bioavailability of these chemicals in sediments depends on the metal chemistry, speciation and sediment properties (Rainbow, 2007).

About sediment properties, taking in count pH and redox potential, the ranges throughout treatments were 6.38 to 7.28 and -202 to -24mV (23mV only at CL treatment) respectively. In addition, the system was aerated to avoid oxygen depletion and death of larvae. According to Chuan et al. (1996) when sediments are aerated, occur an increase of pH and redox potential. Gambrell et al. (1991) investigated the influence of pH and redox potential in the solubility of metals and verified highest metals release at oxidized and acid conditions (400mV and pH 5). After aeration, probably our samples turned to less reduced/or oxidized and alkaline, even in these conditions the authors above cited found low metals solubility.

Other aspect that can influence metals solubility is the sediment particle size and organic carbon content. Silty sediments have lower Cu toxicity compared than sandy sediments. In the lower levels of organic carbon content the metal solubility is higher (Strom et al., 2011). Conservation sites had sandy sediments while Industrial and Farming had sediment with smaller particles (silt to clay). Maybe occurred a compensation of toxicity between conservation and other areas, because of this the responses were similar. BL and GP treatment had the higher levels of organic carbon, probably this fact made a decrease of metals solubility in our samples. Maybe because of these chemical and physical dynamics the metals were not entirely soluble in our samples causing the not expected responses.

For PAHs beyond pH and redox potential, some bacteria are capable to degrade these organic compounds. Benzo(a)pyrene for example, have highest

degradation at oxidized and alkaline conditions (Delaune et al., 1981; Brito et al., 2015) In addition, high total organic carbon levels reduce the bioavailability of hydrophobic organic compounds (Crampon et al., 2014). Our samples had not high levels of total organic carbon, but oxidized and alkaline conditions. These conditions can reduce HPAs in sediments and decrease the toxicity.

Persistent organic pollutants (POPs) like Organochlorine pesticides (OCPs) and Polychlorinated Biphenyls (PCBs) were found in our samples. PCBs were present only at BL treatment (Industrial) above TEL. Regarding OCPs, DDE, DDT and Heptachlor were found above TEL and DDE above PEL in some treatments. Applying the ratio of Strandberg et al. (1998) to DDT/DDE in STA treatment, we can conclude that the input of this pesticide occurred in the past. Aeration can degrade DDT to DDE, probably the DDT content in STA samples was converted to DDE. It is intriguing we did not observe neurotoxicity (decrease of AChE activity) in any treatment due the presence of OCPs. PCBs were found close to urban areas and very consistent with other studies (Barakat et al., 2013).

In a study with *Chironomus dilutus* exposed to contaminated sediments (OPs, organophosphate, pyrethroids, PAHs, PCBs, PBDEs and metals) was proposed to identify the causes of toxicity, and the authors found that pyrethroids were responsible for more than 60% of mortality (Mehler et al., 2011). Pyrethroids were not measured in our samples, maybe were the responsible for the mortality in PCB treatment. In addition, there is a wide range of contaminants that were not measured in this study and could cause other effects in *Chironomus.*

In the annual report, CETESB (2013) taking in count chemical composition (CC), benthic community index (BCI) and ecotoxicity (EC) (*Hyalella azteca*, Ames test and *Vibrio fisheri* Microtox®) classified the quality of sites evaluated in the present study.

RC (Conservation) was considered regular for CC, good for BCI and excellent for EC. In our study, we saw alterations in AChE, ESTs and SOD and histology. PAXA (Conservation) had good (CC), good (BCI) and excellent (EC), for biochemical biomarkers, AChE, ESTs, GST altered and histology.

PEXE (In industrialization process) had good (CC), bad (BCI) and excellent (EC), for biochemical biomarkers, SOD altered, oxidative stress and histology. BL (Industrial) treatment was considered terrible (CC), terrible (BCI) and bad (EC), for biochemical biomarkers, SOD and LPO altered. GP (Industrial) had terrible (CC),

good (BCI), regular (EC), and AChE, GST and SOD altered beyond histological alterations. PCB (Industrial) was classified as regular (CC), good (BCI), terrible (EC), and alterations in ESTs, GST, SOD histology was observed. JAG reservoir had good (CC), good (BCI) and regular (EC), and showed GST and CAT inhibited. CL (Industrial) had good (CC), good (BCI) and excellent (EC) and we observed AChE, ESTs, GST, SOD altered and histopathology.

For farming sites, IS was classified as terrible (CC), good (BCI) and regular (EC), and AChE ESTs, SOD, LPO altered and changes in histology. STA had terrible (CC), regular (BCI) and excellent (EC), it was the worst site with alterations in almost all enzymes except CAT, oxidative stress and histology.

It is possible to observe in CETESB report that there is no clear relationship between chemical composition and benthic community/or ecotoxicity, similarly to our results. Evaluating the sediment quality is a complex task, would be very interesting whether Brazilian environmental agencies start to use biochemical biomarkers in monitoring programs.

When is combined complex mixtures of contaminants and sediments associated with their complex physical and chemical dynamics, is tough to understand how all of these factors will interact with each other and what kind of responses are expected. Single contaminant exposures are very important to understand the effects of single substance and to propose and to help with concentrations threshold established by government laws. But these conditions are difficult to extrapolate to natural samples. Furthermore, studies with more realistic mixtures and their effects in biomarkers are necessary to shed light the understanding of xenobiotics synergism effects.

Chironomids specially *Chironomus sancticaroli* is distributed in São Paulo State, being probably exposed to these sediments. Taking in count the importance of substrate to these organisms, being exposed constantly has some implications to homeostasis of organism and trophic chain dynamics e.g. bioaccumulation.

5. Conclusions

Even after all discussion about interaction of sediments characteristics and chemical contaminants, we guarantee that *Chironomus sancticaroli* was sensitive in different levels when developed in these samples. In all treatments was observed enzymatic alterations, showing stress condition. Santo Anastasio River was the most

altered for biochemical biomarkers. Superoxide dismutase had its activity inhibited in almost all treatments and oxidative stress was observed in Peixe and Santo Anastacio River. Regarding histological biomarkers, fat body was the most sensitive organ in the larvae. Despite these sites are classified as conservation, industrial or farming, no much differences were observed throughout treatments questioning for example the real status of conservation of Claro-Guaçu River and Chavantes reservoir.

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CAPÍTULO II

DISSOLVED OXYGEN COMBINED WITH ARSENIC EXPOSURE IN *Chironomus dilutus* (DIPTERA: CHIRONOMIDAE): IMPLICATIONS TO RESPIRATION AND ANTIOXIDANT RESPONSE*

*This chapter was prepared following Chemosphere instructions for authors

Abstract: In aquatic ecosystems, organisms are exposed to several associations of xenobiotics and physical and chemicals factors. Chironomids are dwelling-sediments larvae that thrive in metals contamination and hypoxia conditions, due to their peculiar physiology. Usually, laboratory experiments do not evaluate the interactions of these conditions. The aim of this study was to evaluate oxygen consumption, hemoglobins (HbA, HbB, HbC and HbD) and GST gene expression in *Chironomus dilutus* exposed to arsenic and hypoxia in single and combined conditions. We exposed for 6 hours in a respirometer equipment (8 chambers - intermittent flow) larvae to four concentrations of dissolved oxygen (100%, 75%, 50% and 5%) and environment relevant concentrations of arsenate (AsV) (0 $\mu g.L^{-1}$, 170 $\mu g.L^{-1}$, 340 $\mu g.L^{-1}$, 680 $\mu g.L^{-1}$) and their combinations resulting in 16 treatments. For oxygen consumption was evaluated only arsenic exposure, and to gene expression all treatments. AsV induced significant oxygen consumption in a concentration-dependent response. The Real time PCR (qPCR) showed that in the presence of AsV the HbA and HBb were induced, while hypoxia induced HbB. Treatments combinations influenced by hypoxia/or arsenic induced responses in HbA, HbD and antioxidant enzyme GST. This is the first report of segregation of specific types of hemoglobin between chemical exposure and hypoxia. Even being considered tolerant to these conditions, chironomids' larvae showed alterations in genetic, antioxidant defense and physiological parameters that can lead imbalance of homeostasis.

Keywords: arsenate, dissolved oxygen, gene expression, respirometry

1 Introduction

The uncontrolled development of human's activities has caused adverse consequences in the environment e.g. water pollution by eutrophication and chemical substances (Gavrilescu, 2010).

Eutrophication increases the amount of organic matter in water, and consequently, it will develop a hypoxia status in the environment (Diaz, 2001). One of these chemical substances is arsenic that is considered a common contaminant in aquatic ecosystems and an EPA priority pollutant (EPA, 2014). This metalloid is found naturally in the environment in high concentrations but, there are some

anthropogenic sources associated with the extraction and use of natural resources such as mining, smelting, and coal combustion (Basu et al., 2014). Both, eutrophication and chemical exposure affect aquatic life and can induce loss of diversity (Marcus, 2004).

Aquatic insects are present in freshwater ecosystems in high diversity and abundance, and this group has typically dominated by chironomids (Diptera, Chironomidae). Chironomids have an important role in energy flow (Berg and Hellenthal, 1992), nutrients and organic matter cycling (Dévai, 1990; Hansen et al., 1997). They have been used in ecotoxicological studies as models e.g. biochemical biomarkers and gene expression investigations (Herrero et al., 2015; Campos et al., 2016). Some species are considered tolerant to chemical substances, e.g. some metals (Odume and Muller, 2011), but the exposure has biological costs and that effects should be elucidated in these organisms due to their ecological importance.

Laboratory ecotoxicological tests often have investigated effects of single compounds or situations. Usually, organisms are submitted in optimal conditions (food, temperature, oxygen, etc.), but such tests do not express the real situation of the environment, where they need to survive in suboptimal circumstances (Holmstrup et al., 2010). Recently, there was an increase of studies that investigated the combination of chemical exposure and abiotic factors such as oxygen depletion for diffent animal groups in general (Ferreira et al., 2008; van der Geest et al., 2002).

Chironomids larvae have a peculiar respiratory strategy based on the presence of hemoglobin in their hemolymph and a switch-on partial anaerobic metabolism. Metabolic rates are related to oxygen uptake in the organisms and can reflect mainly the activity of animals (Mukadam and Kulkarni, 2014). Chironomids need to perform respiratory movements of their body to oxygenate the hemoglobins (Walshe, 1949). Values of oxygen consumption are commonly used in the description of the respiratory capacity, this rate reflects metabolic energy and can be utilized as a precise indicator of the physiological health of the organism (Dalosto and Santos, 2011).

Those respiratory pigments are capable of storing oxygen in high levels of this gas and release it slowly at lower levels (Redecker and Zebe, 1988). Hemoglobins presence is crucial to these organisms to survive in hypoxic sediments (Weber, 1980) but is still not clear the real function of these proteins in chironomids. After the advances of molecular technologies and availability of hemoglobins sequences, no

investigations tested to date, the influence of hypoxia in different types of these globins. In contrast, some studies have shown a possible involvement of hemoglobins in xenobiotics metabolism (Choi and Ha, 2009; Ha and Choi, 2008a, 2008b), so is possible that the same hemoglobin is a multi-function or respiratory and detoxification.

According to Holmstrup et al. (2010) a synergistic pattern between metal exposure and hypoxia could be found in some organisms. Maybe this occurs due to the formation of reactive oxygen species (ROS) at these conditions. Some enzymes compose the antioxidant defense of organisms, e.g. GST (Glutathione *S*-transferase) that inactivate secondary metabolites such as aldehydes, epoxides, and hydroperoxides (Birben et al., 2012).

Understanding differences in gene expression across different types of hemoglobins and antioxidant defense in contaminated and hypoxic water have the potential to shed light on how this considered tolerant organisms to thrive in degraded environments and how their physiology to handle with it. Furthermore our study contributes with which roles hemoglobins are associated with responses to hypoxia and contamination. This study aimed to evaluate respiratory responses and gene expression profiles of hemoglobins and an antioxidant enzyme in single and combined exposure of different dissolved oxygen levels and arsenic.

2 Material and Methods

2.1 Test animals

Egg masses of *Chironomus dilutus* were obtained from a laboratory culture from U.S. Environmental Protection Agency Mid-Continent Ecology Division (EPA), Duluth, MN, US. They were reared under standard laboratory conditions following toxicity tests guidelines (OECD, 2004a, 2004b). Egg masses were transferred to trays (2 L) containing reconstituted were comprised of American Society for Testing and Materials (ASTM) artificial soft water (ASW) (mM:0.57 $NaHCO_3$, 0.17 $CaSO_4 \cdot 2H_2O$, 0.25 $MgSO_4$, and 0.03 KCl; pH 7.8 ± 0.02). Larvae were fed with TetraMin® fish food, as substrate was used quartz sand. Cultures were maintained under constant aeration at 22°C and natural photoperiod.

2.2 Respirometry and hypoxia/arsenic exposure

Experiments were carried out using fourth instar larvae in a respirometer with a 4-channel, fiber-optic-based, intermittent-flow, respirometry system (Loligo Systems, Tjele, Denmark) and AutoResp™ (version 2.1.0; Loligo Systems, Tjele, Denmark) software. The equipment have 8 chambers (1.02-1.54 mL) that were continuously stirred and fitted with a glass spacer ring and stainless steel mesh to separate larvae from a magnetic stir bar. Each chamber measured O_2 consumption rates for 1 *C. dilutus* larva. A peristaltic pump refreshed chamber solutions in programmed 15-min intervals, with each respirometry cycle consisting of a flush/water renewal phase (300 s), a wait/equilibration phase (300 s), and a measurement phase (300 s). Chambers were submerged in an aerated, temperature-controlled water bath (~4 L). Temperature (22°C) was controlled by a programmable heater/chiller (12108-30; Cole Palmer, Vernon Hills, Illinois). A stainless steel heat-exchange coil was immersed in the well of the temperature controller, and a water pump continuously circulated water (5 L/min) from the respirometry water bath through the heat-exchange coil. The experiments consisted in a single exposure of different concentrations of dissolved oxygen (5%, 50%, 75% and 100%) and nominal environment relevant arsenic concentrations (0 $\mu g.L^{-1}$, 170 $\mu g.L^{-1}$, 340 $\mu g.L^{-1}$ (limit permitted by EPA to acute exposure), 680 $\mu g.L^{-1}$) or in combinations, resulting in 16 treatments. In hypoxia treatments, the oxygen level was kept as expected using an oxygen control system in AutoResp™ software. The oxygen control levels were made by a DO (dissolved oxygen) probe and bubbling nitrogen gas from a cylinder attached to the system. To spike water was used arsenate ($HAsNa_2O_4x7H_2O$) obtained from Alfa Aesar (MA, USA).

As a source of exposure, the refreshed chambers solutions were prepared in a 1 L glass beakers submerged in the water bath and connected with the chambers by peristaltic pumps and tubes. In exposure solutions and bath was used ASW filtered by vacuum onto a 0.2 µm Millipore Isopore polycarbonate filter membrane and between each experiment the water bath, pumps, tubes and chambers were rinsed with nitric/acetic acid to avoid microbial activity during the respiratory measurements.

The larvae were subjected to 5 h exposure, and were acclimated to respirometry chambers for 1 h prior to oxygen consumption. The metabolic rates were calculated by averaging oxygen consumption rates across treatments (only

arsenic exposure) and was expressed in (mg O_2/g/h). After measurements, wet weights were obtained to the nearest 0.001g (SartoriusCP124S) and larvae were sacrificed in liquid nitrogen and stored at -80°C freezer to gene expression investigations.

2.3 Chemical measurements

Concentrations of As in the solutions were determined using HPLC-ICP-MS at the Environmental and Analytical Testing Services lab at NC State University. It were taken 3 replicates per treatment.

2.4 Gene expression

Total RNA was isolated following the SV Total RNA Isolation System protocol (Promega; Madison, WI). First strand cDNA was synthesized from the same amount of each total RNA by high-capacity cDNA reverse transcription kit (ABI, 4368814) and all thermocycling was done using a Biorad iCycler. Quantitative Real-Time PCR (qRT-PCR) was used to evaluate the mRNA expression profile of hemoglobins HbA, HbB, HbC, HbD and antioxidant enzyme GST genes; *β*-actin was employed as endogenous gene. Sequences and fragment size of each gene-specific pair of primers are shown in Table 1. q-PCR was performed on an ABI Prism 7700 Sequence Detection System (Applied Biosystems (ABI); Carlsbad, CA) using default parameters. Amplification mixtures consisted of 5 µL of SYBR Green Master Mix (Applied Biosystems (ABI); Carlsbad, CA), 10 µM primers, 20 ng template cDNA and nuclease free water in a total volume of 10 µL. qRT- PCR conditions were 2 min at 94°C, followed by 40 cycles at 95°C for 30s, 60°C for 30s, and 72°C for 30s. The relative expression of each amplicon was calculated by the corrected delta delta Ct method (Pfaffl, 2001), with all expression normalized to Actin-rRNA levels in initial control samples. Relative levels of Actin were confirmed to be approximately equal across all treatments.

2.5 Statistical analysis

The data were tested to the distribution pattern of the residue by the Shapiro–Wilk test, and the homogeneities of variances by the Brown–Forsy test. In some cases log transformation was needed. For oxygen consumption, hypoxia, arsenic exposure and combinations effects, the comparisons were performed using ANOVA-one way followed by post hoc Tukey test.

Table1: Primers for each gene evaluated and the expected amplicon size.

Gene	Primer	Fragment size
HbA-forward	5'- TCGCTGGAAAGGATGTTG -3'	109 bp
HbA-reverse	5'- GCTGCGTTTCCAGTAAGT -3'	109 bp
HbB-forward	5'- CCAGCGAATTGTGGGAAA -3	100 bp
HbB-reverse	5'- GATCATCATGGGATCAGGTTAG - 3	100 bp
HbC-forward	5'- GCAGCCATTTCGTTGATAAG -3'	105 bp
HbC-reverse	5'- CACACATGCCGGAAGAAT -3	105 bp
HbD-forward	5'- CGAAGATAGCGTCGAAGATG -3	102 bp
HbD-reverse	5'- GTGCCTCAATGACCTCATAC-3	102 bp
GST-forward	5'- CACTTGGGCTGATGTCTATG-3	121 bp
GST-reverse	5'- CTCAGCTGCCATTACTTTCT-3	121 bp
β-actin-forward	5'- CCTCCCTTGAGAAGTCCTAT-3	123 bp
β-actin-reverse	5'- GGATACCGCAAGATTCCATAC-3	123bp

3 Results

The chemical measurements of arsenic showed the values above nominal concentrations, in control groups the values were <0.1 $\mu g.L^{-1}$, the averages were C1= 209 $\mu g.L^{-1}$, C2= 440 $\mu g.L^{-1}$, C3= 836 $\mu g.L^{-1}$.

Looking at the oxygen consumption, after 6 hours of arsenic exposure there was an increase of metabolic rates at the treatments. The response was concentration-dependent with higher consumption at 680 $\mu g.L^{-1}$ ($F_{3,20}$=27.29; p<0.0001) (Figure 1).

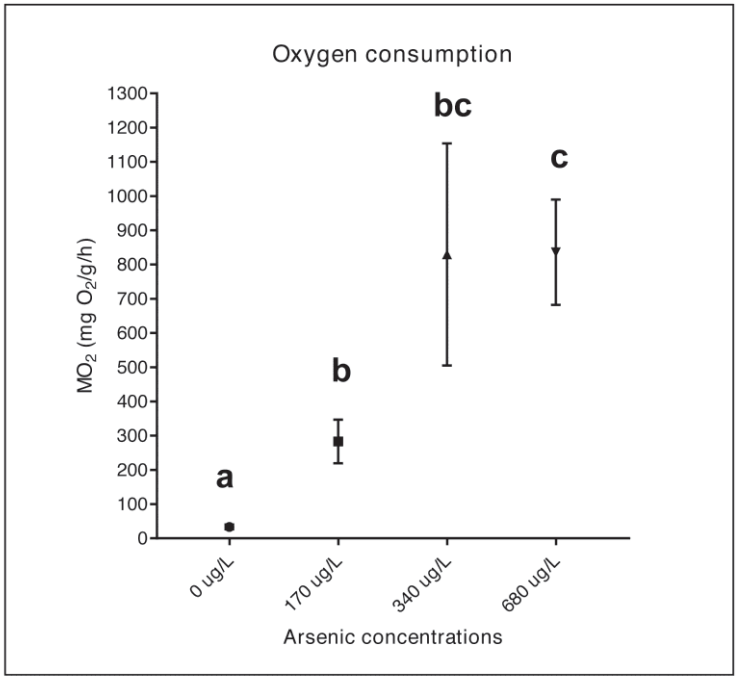

Figure 1: Oxygen consumption in *Chironomus dilutus* exposed do arsenic in four concentrations. Values are expressed as mean ± standard error of mean. Different letters indicates significant statistical differences between treatments. ANOVA-one way with Tukey test *a posteriori* ($p<0.05$).

Throughout the 16 treatments, for HBs A, B, C, D and GST was not possible to have results from “340 µg.L^{-1} arsenic + 50% of oxygen” and "680 µg.L^{-1} arsenic + 50% of oxygen" beyond "340 µg.L^{-1} arsenic + 70% of oxygen" in HbD.

Considering the gene expression profile, it was possible to observe some alterations caused by arsenic and/or hypoxia. In the Hb A, comparing the treatments that tested just hypoxia (0 µg.L^{-1} of arsenic), effects were not observed between control and treatments (Figure2a). In treatments that evaluated only arsenic effects (100% of oxygen), was observed an upregulation in 340 µg.L^{-1} ($F_{3,13}$=3.30; p=0.02) (Figure2b). The treatments 50% of oxygen+no arsenic and 100% of oxygen+170 µg.L^{-1} did not show effect, but the combination of these treatments 50% of oxygen+170 µg.L^{-1} upregulated the expression ($F_{3,41}$=0.63; p=0.0006) (Figure 2c).

For HbB, hypoxia increase the expression in 50% of oxygen and a downregulation was observed in 5% ($F_{3,13}$=0.2936; p<0.0001) (Figure 2d). Arsenic also altered the expression of this globin, an increase was observed at lower

concentration 170 µg.L^{-1} ($F_{3,10}$=0.055; p=0.0204) (Figure 2e). The combinations did not show differences ($F_{12,31}$=0.802; p=0.0548) (Figure 2f).

In the HbC, hypoxia ($F_{3,13}$=0.633; p=0.02525) (Figure 2g) and arsenic ($F_{3,10}$=2.29; p=0.4496) (Figure 2h) did not show effects in gene expression but the combination 75% of oxygen+170 µg.L^{-1} had a decrease of gene expression when compared with just 75% of oxygen or just 170 µg.L^{-1} ($F_{13,33}$=1.033; p=0.0407) (Figure 2i).

About HbD, it was observed the same pattern of HbC, hypoxia ($F_{3,11}$=1.67; p=0.09) (Figure 2j) and arsenic ($F_{3,10}$=0.27; p=0.37) (Figure 2k) did not cause effects. The combination 75% of oxygen+170 µg.L^{-1} showed an increase of expression when compared with 50% and no arsenic, and 100%, 75%, 50%+170 µg.L^{-1} had differences with 50%, 50%, 5%+170 µg.L^{-1} ($F_{12,26}$=0.73; p<0.0001) (Figure 2l).

Regarding the antioxidant defense, GST has not shown effects by hypoxia comparing with control, but there were differences between treatments ($F_{3,15}$=0.46; p=0.048) (Figure 2m). Similarly, arsenic not caused effects when compared with control but there was effects between treatments ($F_{3,13}$=0.89; p=0.031) (Figure 2n). Interestingly, there were alterations of expression in combinations related to hypoxia and arsenic. There were significant differences between 100%, 75% and 5% and 50% of oxygen+170 µg.L^{-1} and 50%, 50% and 5% of oxygen+170 µg.L^{-1} beyond 5% of oxygen and no arsenic and 5% of oxygen + 680 µg.L^{-1} ($F_{13,42}$=1,92; p<0.0001) (Figure 2o).

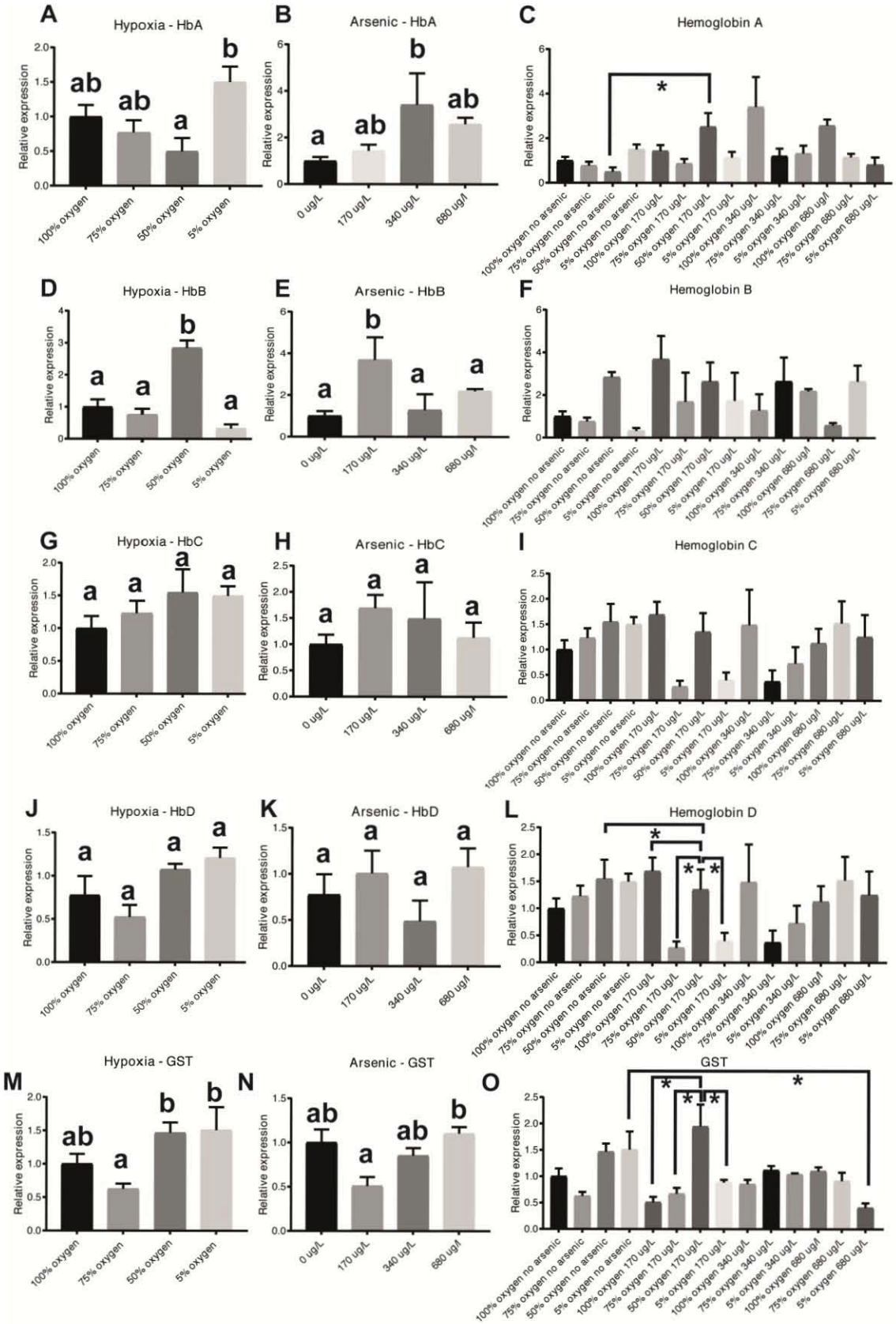

Figure 2: Gene expression profiles in *Chironomus dilutus* exposed to hypoxia and arsenic. A-C: Hemoglobin A, D-F: Hemoglobin B, G-I: Hemoglobin C, J-L: Hemoglobin D, M-O: GST enzyme. Values are expressed as mean ± standard error of mean. Different letters/or brackets indicates significant statistical differences between treatments. ANOVA-one way with Tukey test *a posteriori* ($p<0.05$).

4 Discussion

Chironomids larvae were considered a good model for physiological studies since 40's within insects, mainly due to their peculiar respiratory physiology (Walshe, 1949). Some studies regarding to hemoglobins functions and larvae respiratory behavior contributed to understanding the mechanisms involved to hypoxia tolerance in these organisms (Weber et al., 1985; Zebe, 1991). In the fourth instar 90% of free proteins in the larvae hemolymph are hemoglobins (English, 1969).

Regarding oxygen consumption, it has been detected an increase of metabolic rates in the treatments. The gastropod *Nassarius obsoletus* showed a decrease of oxygen consumption in arsenic exposure (MacInnes and Thurberg, 1973). For other metals e.g. cadmium, chromium, copper, zinc in other aquatic organisms was reported only decrease of oxygen consumption (Thurberg et al., 1973; Cheung and Cheung, 1995; Vutukuru, 2005). It is not clear how arsenate could increase the metabolic rates in *C. dilutus.*

According to some authors the hemoglobins titers can be influenced by several natural factors, e.g. age of larvae (Vafopoulou-Mandalos and Laufer, 1982), molting hormones (Vafopoulou-Mandalos and Laufer, 1984), seasonality, depth in sediment (Osmulski and Leyko, 1986). We used in our experiments controlled conditions of temperature and photoperiod beyond age of larvae to avoid the influence of these parameters.

It was observed influence of hypoxia just in hemoglobin B when compared with control, an increase at 50% of oxygen. The hemoglobins in Chironomidae differently of mammals hemoglobins, not just transport oxygen, but play a role of storage in higher pressures of this gas and release it slowly in lower pressures (Redecker and Zebe, 1988). At 50% of oxygen probably there is a signal to produce more hemoglobins in order to store more oxygen. In contrast, at 5% no more much oxygen is available to bind to globins, justifying the return to basal levels of titers in the hemolymph. In hypoxia conditions (~5%) stored oxygen can hold the aerobic metabolism, but when the organisms face to severe hypoxia (<5%) they are capable to switch to anaerobic metabolism, producing alcohol (alcoholic fermentation) as metabolite and not the common toxic lactate, this fact allows larvae to survive to long periods in anoxia (Grazioli et al., 2016). In a study evaluating the total hemoglobins contents and alcohol dehydrogenase (ADH) activity in *Chironomus riparius* exposed

to progressive hypoxia and anoxia, was observed the same trend when compared with our results. An increase around 60% and decrease at 5% of hemoglobins content beyond increase of ADH activity at 5% demonstrating the switch-on to anaerobic metabolism (Grazioli et al., 2016).

We quantify the expression for four hemoglobins and only HbB shows response to hypoxia. There is a high polymorphism to chironomids' hemoglobins being already described up to 16 types, probably there is more types responsible to oxygen storage (Weber et al., 1985).

Considering the arsenic exposure, there was an upregulation to Hbs A, B. Indeed, it is clear that there is some relationship between chemical exposure and hemoglobins titers, but we still don't know whether this phenomenon is direct or indirect. Some studies showed alterations in hemoglobin expression in exposure to pesticides, polycyclic aromatic hydrocarbons and metals. Exclusively about metals, lead and cadmium showed effects in hemoglobin expression. The metal lead in *C. dilutus* increase HbA and increase/and decrease HbB while in *C. riparius* increase the HbA and C. The cadmium in *C. dilutus* increase the expression of HbIIB while in *C. riparius* increase the HbA, B, C, D and E (Choi and Roche, 2004; Lee et al.,2006; Anderson et al., 2008; Ha and Choi, 2008a, 2008b; Lee et al., 2008; Choi and Ha, 2009; Lee and Choi, 2009). Hemoglobins have a related pseudoperoxidase activity or monooxigenase similar to cytochrome P450 reductase, the globins structure seems like to P450 due to the presence of heme group (Osmulski and Leyko, 1986).

Regarding to the antioxidant GST enzyme, there is no direct effect in hypoxia and arsenic exposure when compared with controls, but there were alteration within treatments. Furthermore in combinations both, hypoxia and arsenic had effects on gene expression. Arsenic can induce the ROS formation because dimethylarsine can reacts with molecular oxygen to generate a dimethylarsenic radical and superoxide anion. The addition of another molecule of molecular oxygen onto the dimethylarsenic radical results in a dimethylarsenic peroxyl radical. A hydroxyl radical is generated during these reactions via involvement with cellular iron and other transition metals (Liu et al.,2000; Ercal et al., 2001). Arsenic increase GST levels in *Musca domestica* (Diptera: Muscidae) and *Trichoplusia ni* (Lepidoptera: Noctuidae) more specifically arsenite, arsenate not showed effects to this enzyme (Zaman et al., 1995). There is no data in the literature showing hypoxia effects on

GST enzyme, but probably this occurs because low levels of oxygen induce the ROS formation (Guzy et al., 2005).

Chironomids have a peculiar respiratory strategy that is so far to be fully comprehended. This very adapted physiology allow theses organism to thrive in challenging conditions. We could demonstrate for the first time segregation of functions by specific hemoglobins. HbA only responded directly to arsenic and HbB responded to both, C and D not show effects of hypoxia and arsenic in a single exposure. In the environment the chironomids are exposed to complex mixtures of xenobiotics associated with several physic-chemical factors. It would be interesting to investigate in next studies the interaction of all variables in respiratory responses. In addition, there is still a lack of biochemical studies demonstrating the direct binding of hemoglobins and xenobiotics to prove the real role of these globins in xenobiotics metabolism.

5 Conclusions

Although the chironomids' larvae are considered tolerant to chemical and hypoxia, the biomarkers already described in the literature showed effects to these conditions. The metabolic rates were very increased in the highest concentration (680 $\mu g.L^{-1}$). Hemoglobins demonstrated segregation of function e.g. HbA upregulated in arsenic exposure, HbB showed responses to hypoxia and arsenic exposure while HbC and D just had alterations in the combinations of these conditions. The antioxidant enzyme GST, change the gene expression levels to arsenic and interestingly to hypoxia. In summary, different levels of stress were showed; in the ecosystem larvae can face with more complex combinations of environmental degradation, which can limit the fitness of these organisms.

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CAPÍTULO III

BIOCHEMICAL RESPONSES ACROSS CHIRONOMIDAE (DIPTERA) SPECIES EXPOSED TO INSECTICIDES: DO SELECTION PRESSURE OR EVOLUTIONARY HISTORY DRIVE THE TOLERANCE?*

*This chapter was prepared following Aquatic toxicology instructions for authors

Abstract: The application of insecticides to order the control of agricultural pests and disease vectors can affect non-target organisms, as chironomids. The tolerance of species is associated with some enzymes and the response level is related to shared environmental conditions or evolutionary history. The aim of this study was to evaluate the drivers of biochemical responses across chironomids species exposed to pesticides using an evolutionary approach. It was collected from eggs, twelve species of chironomids and fourth instar larvae were exposed to 0.056 $mg.L^{-1}$ of malathion in bioassays with controlled conditions and was measured AChE (acetylcholinesterase), EST-α (alpha esterase) EST-β (beta esterase) or homogenates from control larvae were exposed to propoxur in a microplate assay. To compare the responses we applied the *K* and λ index to estimate phylogenetic signal using a molecular phylogeny (COI gene) generated in our study. Furthermore, to compare the responses at same species collected at different sites to detect selection pressure influence. It was observed a statically significant inhibition of AChE in different levels in almost all species, for ESTs different behaviors were observed (decrease or increase of activity). No phylogenetic signal was detected to enzymes responses in the family level, to AChE and EST-α a trend, but not significant signal was identified to *Chironomus* genus. The selection pressure influence was detected because were observed different responses in the enzimatic activity in organisms of same species collected at different sites, being resistant that ones from intense pressure locations. Furthermore, metabolism in control organisms from sites with agricultural influence had lower levels of AChE and ESTs when compared with control organisms from sites wothout agricultural influence. AChI assays showed that *Goeldichironomus* species are more sensitive than *Chironomus* species. Chironomids natural populations are suffering with selection pressure having a resistance framework, this status can decrease the fitness of organisms showing populational problems a long-term.

Keywords: phylogenetic signal, selection pressure, biochemical biomarkers, pesticides

1 Introduction

Chironomids are considered the most abundant and widespread insects in aquatic ecosystems. Their worldwide diversity is estimated around ~20,000 species (Merrit et al., 2007). The physiological plasticity in these animals allows them to thrive in a wide range of habitats. It is reported by several authors the high tolerance of chironomids to pollutants and hypoxia, beyond the presence of hemoglobins in the hemolymph (Choi et al., 2001).

From the evolutionary point, something intriguing happened in the chironomids evolution. Their sisters groups Ceratopogonidae and Simuliidae larvae have not high tolerance to environmental degradation, hemoglobins and are hematophagous (Miller et al., 1997; Nel and Azar, 2012). Adults do not feed and live around five days, being the larval stage the main target of contaminants in water bodies.

The tolerance of chironomids had been reported to be variable in different genera (Hilsenhoff, 1987). However, even species in the same genera show variable levels of tolerance, and some responses are conserved or not (Carew et al., 2011). Within organisms, physiological traits are kept in a descent-based genetic process and the environment will select them (Poteat et al., 2015). Understanding the responses of species closely related help us to have predictive power to estimate tolerance of certain clades.

Comparative approaches had remarkable improvements after incorporation of evolutionary perspectives. A way to measure the strength of traits similarity is using phylogenetic signal metrics. When a phylogeny is available, it is possible to use the *K* index (Blomberg et al., 2003) or λ index (Pagel, 1999) that allows us to make an estimate and to test of phylogenetic signal in a cladogram. In these indexes a $K/\lambda = 1$ means that a trait has a strong phylogenetic signal in closely related species. Values of $K/\lambda < 1$ mean that closely related species are more different from each other than expected. Using the *K* index, Carew et al. (2011) detected a strong phylogenetic signal for zinc tolerance in some Chironomidae genera, taking in count their presence/absence in contaminated sediments. Ignoring evolutionary context in comparative data sometimes can lead misinterpretations conclusions (Chiari et al., 2015)

Tolerance/sensitivity is a result of organism traits related with uptake and metabolism of pollutants (Guénard et al., 2011). Traits can come from two distinct histories, ecological or evolutionary. Ecologists address the response level with distribution and shared environmental conditions while evolutionary biologists link the responses with a context of lineage (Garland et al., 2005; Poff et al., 2006).

Using an evolutionary context in ecotoxicology with different taxa (vertebrates and invertebrates), there was detected phylogenetic patterns in traits as metals bioaccumulation (Buchwalter et al., 2008; Poteat and Buchwalter, 2014), metals efflux (Poteat et al., 2013), tolerance to metals, insecticide, herbicides (Carew et al., 2011; Hammond et al., 2012; Larras et al., 2014; Chiari et al., 2015; Malaj et al., 2016) mass, time to hatching, time to metamorphosis, and frequency of abnormalities (Egea-Serrano et al., 2012). Considering the metabolism in response to chemical exposure, there is only one paper showing phylogenetic signal in esterases (butyrylcholinesterases and carboxylesterases) of digestive tract in species of passerine birds from Chile inhabiting agricultural areas, but this study is not related to tolerance (Narvaez et al., 2015).

In agricultural areas destined for intensive production of food, pesticides (organophosphates, pyrethroids, carbamates, organochlorines) are intensively applied in the environment in order to eliminate, preventing, repealing any pests or in cities against vectors of diseases (Luc et al., 2016). But non-target arthropods as chironomids can be affected by these organic pollutants (Rebechi et al., 2014).

Specifically, malathion (organophosphate) and propoxur (carbamate) are insecticide considered highly toxic to aquatic organisms. These insecticide is applied in agrosystems and in vectors control programs and have a wide use in the world (WHO, 2009).

Related to toxicity and metabolism biomarkers in exposure specifically to malathion, basically four esterases enzymes are involved, i. e. acetylcholinesterases (AChE), butyrylcholinesterases (BChE), vertebrates have both, but arthropods have only AChE. Carboxylesterases (CbE – EST-alpha and EST-beta) and phosphotriesterases (PTE) (Toutant, 1989; Fukuto, 1990)

AChE a neurotoxicity biomarker, plays a role in the degradation of acetylcholine neurotransmitter at synaptic gap between neuron cells (Lang et al., 2012). It is usual to observe the inhibition of this enzyme in pesticides and metals exposure scenario (Frasco et al., 2005; Rebechi et al., 2014). The intensive selection

pressure by pesticides application (e.g., organophosphates and carbamates) can make the natural populations to contain individuals with AChE less sensitive to pesticides (Fournier and Mutero, 1994).

In addition, carboxylesterases (CbE) biotransformation biomarkers, are phase I enzymes enable to bind to pollutants and hydrolyzing ester bonds to generate one molecule of acid and alcohol as metabolites (Montella et al., 2012). Regarding the esterases behavior, both induction and decrease can be observed in exposed to a wide range of xenobiotics. Furthermore, the increase of esterases activity may be associated with resistance framework (Wheelock et al., 2005).

The high tolerance in some chironomids is still intriguing, evaluating how biochemical biomarkers related to pesticide exposure across species behave, can help us to shed light who are the main drivers of these responses, evolution or selection pressure. This is the first attempt to show an evolutionary approach in biochemical biomarkers in aquatic toxicology. This study aimed to evaluate the biochemical responses of chironomids species exposed to pesticides, using an evolutionary approach.

2 Material and methods

2.1 Chironomids sampling, culture and identification

In order to obtain several larvae of same species without any pesticide exposure, we worked with chironomids egg masses. To have eggs, we did active collection by aspiration of females and males of chironomids, using a hung fluorescent light in front of a white sheet (light-trap) close to some water body as attractive. The adults were replaced in a cage containing a tray with water from the closest water body and vegetation as substrate to oviposition. The sampling sites are shown in the Figure 1.

It was possible to obtain egg masses (120 egg masses) from twelve species of Chironomidae belonging to Chironominae subfamily, Chirinomini tribe, *Goeldichironomus, Chironomus* and *Dicrotendipes* genera. The organisms were from almost all collecting sites, except Ilha do Mel and Porto Rico (Parana State), where adults were collected but we did not obtain egg masses (Table 1). We also used *Chironomus sancticaroli* species from a laboratory colony.

After oviposition, they were reared under standard laboratory conditions following toxicity tests guidelines (OECD, 2004a, 2004b). Egg masses were transferred to trays (2 L) containing reconstituted water with 1.2 mg L-1 hydrated CaSO4, 0.08 mg L-1 KCl, 2.44 mg L-1 MgSO4x7H2O, and 1.92 mg L-1 Na2CO3, conductivity of 160 µS cm-1, pH 7.2 and hardness 16 mg L-1. Larvae were fed TetraMin® fish food, as substrate was used quartz sand 50-70 mesh (Sigma-Aldrich®). Cultures were maintained under constant aeration at 25 °C and standard light–dark periods 12:12. Trays were covered to avoid cross contamination with other species. When the larvae reached the fourth instar (F0), some of them were used in the bioassays, other collected for identification. The resting organisms were used as adults for identification (males) and molecular studies (females).

To fixate and preparing of specimens, slides were mounted following (Trivinho-Strixino et al., 1995) to larvae and (Cranston et al., 1989) to males. The identification process was accomplished together with Dr. Susana Trivinho Strixino, a Brazilian chironomids taxonomist to guarantee the correct species names. Voucher specimens will be deposited in Padre Jesus Santiago Moure collection, Departamento de Zoologia,UFPR.

2.2 Bioassays

For exposure was used malathion Pestanal® (purity > 96,1%, CAS number 121-75-5, Sigma-Aldrich catalog nº 36143).

The fourth instar larvae were exposed to 0.056 $mg.L^{-1}$ (for analytical detection of concentration was used mass spectroscopy with 50µl of direct infusion of each aliquot in a 50µl/min of constant flow, samples were taken in the T0 and Tf). This concentration represents the malathion CL_{25} to *Chironomus sancticaroli*, the tropical chironomid species usually kept in laboratory to ecotoxicological studies in Brazil, this concentration was adopted to guarantee effects on biochemical biomarkers (Rebechi-Baggio, 2016). Larvae were exposed for 48 hours in a tree replicates design with at least five larvae, using 13 g of quartz sand (50-70 mesh - Sigma-Aldrich®) as substrate and 100mL of solution in a plastic container. It was carried out a control and ethanol solvent groups as well. Experiments were kept in controlled temperature and photoperiod conditions (25°C and 12 light:12 dark).

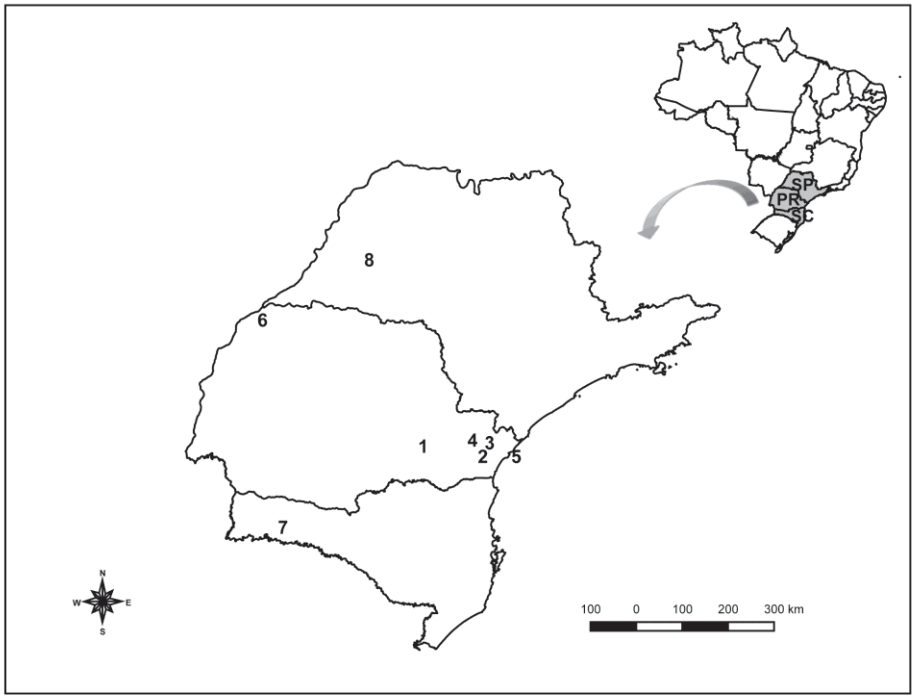

Figure 1: Collecting sites of chironomids. 1-Palmeira-PR (25º25'44"S, 50º00'21"W); 2-Morretes-PR (25°28'37"S, 48°50'02"W), 3- Antonina-PR (25º19'15''S, 48º42'24''W), 4- Bocaiúva do Sul-PR (25°14'24"S, 49º02'03"W), 5- Ilha do Mel-PR (25°33'7.9"S, 48°18'1.2"W), 6- Porto Rico-PR (22°45'55.6"S, 53°15'25.8"W), 7- Chapecó-SC (27°02'51.9"S, 52°37'16.7"W) and 8- Lucélia-SP (21°43'35.1"S,50°59'47.3"W).

Table1: Chironomidae species collected and the specific sites of sampling.

Species	Locality
Goeldichironomus luridus Trivinho-Strixino &Strixino, 2006	Palmeira-PR
Goeldichironomus neopictus Trivinho-Strixino &Strixino, 1998	Lucelia-SP
Goeldichironomus neopictus Trivinho-Strixino Strixino, 1998	Chapecó-SC
Goeldichironomus maculatus Trivinho-Strixino Strixino, 1991	Palmeira-PR
Goeldichironomus holoprasinus Goeldi, 1905	Palmeira-PR
Goeldichironomus holoprasinus Goeldi, 1905	Morretes-PR
Goeldichironomus pictus Reiss, 1974	Morretes-PR
Chironomus sancticaroli Strixino-Strixino, 1981	Palmeira-PR
Chironomus calligraphus Goeldi, 1905	Antonina-PR
Chironomus calligraphus Goeldi, 1905	Lucélia-SP
Chironomus calligraphus Goeldi, 1905	Morretes-PR
Chironomus stigmaterus Say, 1823	Morretes-PR
Chironomus stigmaterus Say, 1823	Chapecó-SC
Chironomus fittkaui Correia & Trivinho-Strixino, 2007	Morretes-PR
Chironomus sp1	Chapecó-SC
Chironomus sp2	Bocaiúva do Sul-PR
Chironomus sp2	Antonina-PR
Dicrotendipes soccus Epler, 1988	Chapecó-SC

2.3 Biochemical Biomarkers

Each sample represents an individual larva (final 4th instar). The sample was homogenized in 330µl of Milli-Q water, and centrifuged at 12000 x g for 1 minute at 4°C. The supernatant was aliquoted in plates in a specific volume for each biomarkers and storage at -80°C freezer.

Each enzymatic assay was performed in triplicate, and measured at SpectraMax 190 Absorbance Microplate Reader (Molecular Devices®).

Acetylcholinesterase activity AChE *in vivo* was measured spectrophotometrically at 405 nm, following Ellman et al. (1961), modified to microplate by Silva de Assis (1998). The assay was carried out using 25 µl of sample, 200 µl of DTNB 0.75 mM, 50 µl of ATC 10 mM. It was incubated for 30 minutes and the kinetic was read each one minute for five minutes. The results were expressed in mmol/mg protein/min.

Acetylcholinesterase AChI *in vitro* (protocol to measure AChE inhibited- *Aedes aegypti* protocol) was carried out to eliminate the influence of different uptake rates across species using the control groups homogenates. This experiment consisted in an application of the propoxur (carbamate insecticide) in the plate, added to ATC solution. The final concentration in the well was 4.20 $mg.L^{-1}$ of propoxur and was measured as endpoint at 405 nm without incubation. The results were expressed in % of inhibition. (Valle et al., 2006).

Alpha and beta esterases (EST-α, EST-β) were measured according to Valle et al. (2006) at 570 nm. It was used 10 µl of sample and was added 200 µl of apha/or beta-naphthil acetate/Na phosphate 0.3 mM. The assay was incubated for 15 minutes, and then 50 µl of Fast Blue dye 0.3% was added and five minutes of incubation again. The results were expressed in alpha/or beta naphthol/mg protein/min.

2.4 DNA extraction, PCR and sequencing

DNA extraction was performed to investigate barcoding sequences from females by Bona et al. (2012). Conventional PCR was run to amplify cytochrome oxidase subunit I, using the primers LCO1490 (5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3') and HCO2198 (5' - TAA ACT TCA GGG TGA CCA AAA AAT CA -

3') (Folmer et al., 1994). We used Taq DNA polymerase from Sigma-Aldrich®, all reactions used 1 µl of DNA template in standard protocols for 25 µl of reaction. The purification reaction was performed with the QIAquick PCR Purification Kit (Qiagen®). The sequencing was made at Center for Human Genome Studies (University of São Paulo-São Paulo/SP) using an ABI 3730 DNA Analyzer (Applied Biosystems®). Sequences obtained were edited in BioEdit, version 7.2.5 (Hall, 1999) and aligned with Muscle algorithm of Mega software, version 6 (Tamura et al., 2013). Consensus sequences were compared with available chironomids sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/), using the tool Blast N. The sequences will be deposited in GenBank.

2.3 Phylogenetic analysis

The similarity's topology of Chironomidae was performed using *Simulium* sp. (Diptera: Simuliidae - sister group of Chironomidae) as an outgroup táxon . Prior to Bayesian Inference (BI) analyses the optimum nucleotide substitution model for individual codon partitions for each dataset was identified with the Akaike Information Criterion (Akaike 1973) implemented in jModeltest 3.7 (Posada and Crandall 1998). The best substitution model was GTR+I+G. For data, Bayesian Inference (BI) was run using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) where the best topologies were found using the MCMC algorithm. A total of 1000000 generations were run and the burn-in for the analysis was 65.

2.4 Statistical analysis

To compare enzymatic activities between the control and treatment within same species, an Unpaired Student t-test was performed, after log transformation in some cases or Mann-Whitney, non-parametric test for data without homogeneity. The comparison of % of inhibition in AChI across species was performed by Kruskal-Wallis with Dunn's test *a posteriori*.

The phylogenetic signal for each enzyme was calculated by *K* statistic (Blomberg et al., 2003) or λ index (Pagel, 1999) with a *p* value associated performed in R Development (Core Team, 2011). It was used in the analyses the delta (treatment average – control average) of enzymes activities across species to

compare the responses or % of inhibition for AChI. The phylogenetic signal was tested to all species and within *Chironomus* genus.

For enzymatic comparison and phylogenetic signal same species collected at different sites were considered together. To sites comparison, the species were analyzed separately.

3 Results

The real concentration of malathion detected in initial time was 0.0462 $mg.L^{-1}$ very similar with nominal concentration. After 48 hours the concentration was 0.011$mg.L^{-1}$, being possible to observe a reduction of 75% of the initial concentration. In the bioassays with malathion, the species *G. maculatus* and *Chironomus* sp2 from Bocaiúva do Sul did not survive at treatment, being not possible to analyze enzyme activities. In controls larvae at propoxur assays, we could analyze AChE inhibition for these species. For AChE at malathion exposure we had statically significant inhibition of its activity to *D. soccus* ($F_{11,9}$= 1.057; p=0.0189), *G. holoprasinus* (U=100; p=0.0012; df=22,19), *G. neopictus* (U=87; p=0.02; df=16,17), *Chironomus* sp1 ($F=_{2,8}$=35.51; p<0.001), *C. fittkaui* (U=0; p=0.004; df=3,7), *C. sancticaroli* ($F_{17,18}$=2.34; p=0.0029), *C. calligraphus* ($F_{12,12}$=2.73; p<0.0001) and *C. stigmaterus* (U=0; p<0.0001; df=12, 7) (Figure 2a).

For AChI at propuxur assay in the microplate, we had statically significant difference across % of enzyme inhibition between species (H(10, N=123)= 72.96; p= 0.0000). There was a trend of difference between some species of *Goeldichironomus* and *Chironomus. Goeldichironomus* in general (except *G. maculatus*) had their average of inhibition above 80% and *Chironomus* had below 80% (Figure 2b).

The esterases had a different behavior across species, we observed both statically significant increase and decrease of their activity. For EST-α, it was observed an increase of activity in *D. soccus* ($F_{11,9}$=1.68; p=0.0156), *G. luridus* ($F_{7,9}$=1.95; p=0.0067) and a decrease in *G. holoprasinus* (U=76; p=0.0005; df=19,19), *Chironomus* sp2 ($F_{2,8}$=4.10; p=0.0021) and *C. stigmaterus* ($F_{12,9}$=2.74; p=0.01) (Figure 2c). For EST-β, it was observed an increase in *G. neopictus* (U=56; p=0.0006; df=19,15) and a decrease in *G. holoprasinus* ($F_{19,23}$=1.118; p=0.01), *C. calligraphus* ($F_{16,17}$=2.29; p<0.0001) and *C. stigmaterus* ($F_{7,13}$=2.008; p=0.0088) (Figure 2d).

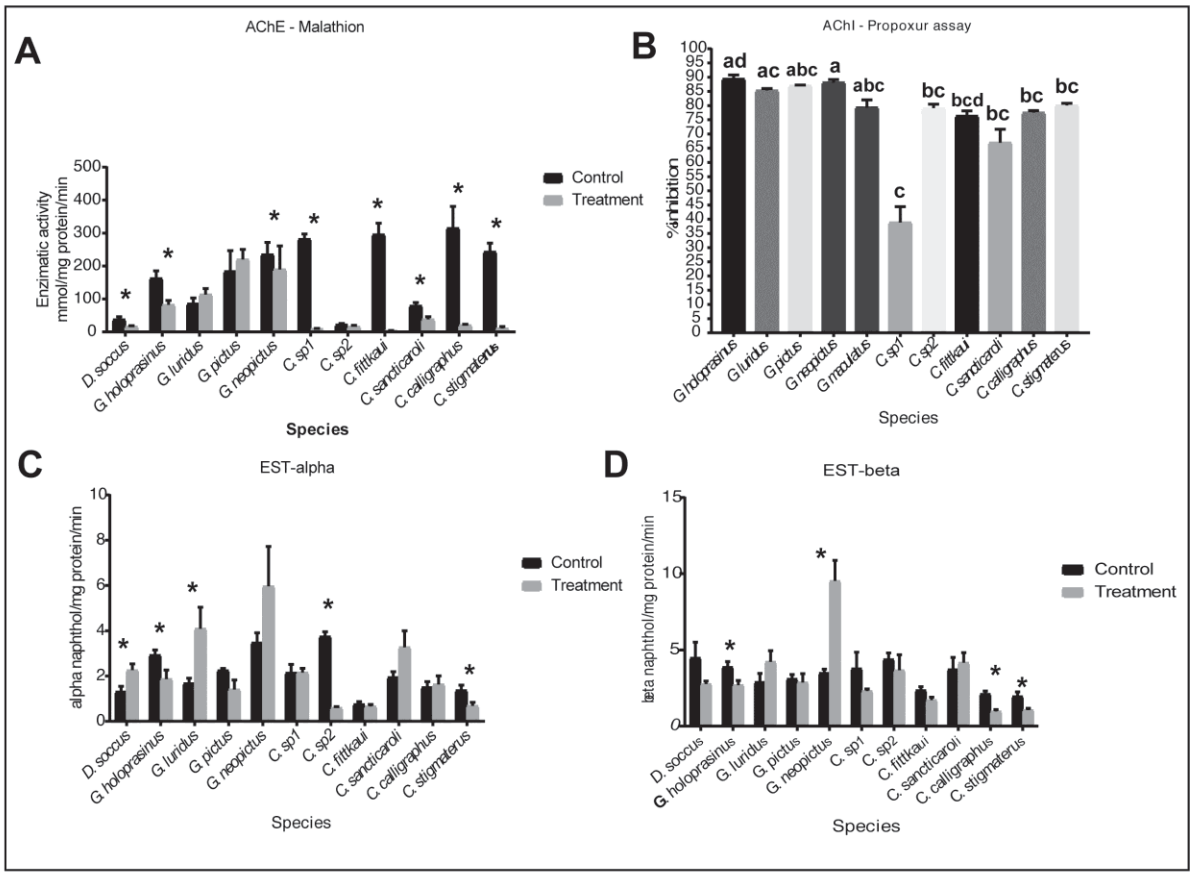

Figure 2: Enzymatic responses of chironomids across different sprecies exposed to malathion/or propoxur. A: AChE (acetylchilinesterase), B: AChI (acetylcholinesterase, propoxur assay), C: EST-α (alpha-esterase) and D: EST-β (beta esterase). Values are expressed as mean ± standard error of mean. Asterisks/or letters indicate statistically significant differences ($p < 0.05$) compared to control group or across species; Student t-test/or Mann-Whithney/or Kruskal-Wallis.

The phylogenetic reconstruction shows *Chironomus* genus as a monophyletic group, it allows us to test phylogenetic signal within this group beyond all species. For malathion exposure, in the AChE activity we observed a weak phylogenetic signal for all species in both metrics (K= 0.53; p=0.24 and λ= 0.00007; p=1), for *Chironomus* no phylogenetic signal was observed, but there was a trend due to high values of K and λ (K= 0.95; p=0.09 and λ= 0.99; p=0.14) (Figure 3a).

Regarding AChI (propoxur assay) a weak phylogenetic signal was observed in both, all species (K= 0.23; p=0.71 and λ= 0.25; p=0.60) and *Chironomus* (K= 0.21; p=0.72 and λ= 0.00006; p=1) (Figure 3b)

In the EST-α no phylogenetic signal was observed for all species (K= 0.33; p=0.61 and λ= 0.00006; p=1) while a trend was detected to *Chironomus*, but also with no significant p value (K= 0.71; p=0.22 and λ= 0.76; p=0.12) (Figure 4a) and for EST-β a weak signal was observed in both, all species (K= 0.25; p=0.71 and λ= 0.00006; p=1) and *Chironomus* (K= 0.32; p=0.65 and λ= 0.067; p=0.91) (Figure 4b).

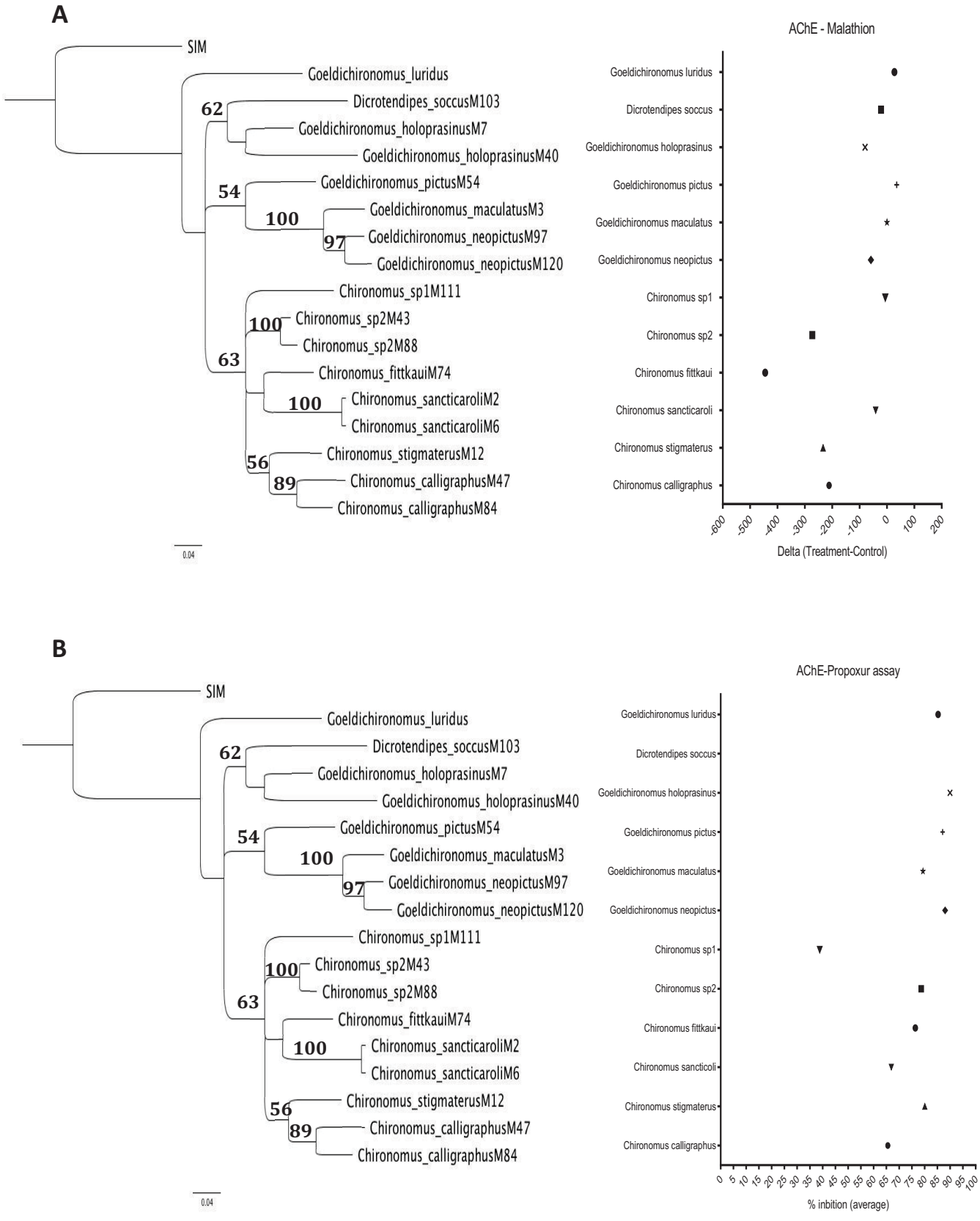

Figure 3: Phylogenetic relationships between species and enzymatic response level (treatment average-control average) of chironomids. A: AChE (Acetylcholinesterase) – malathion exposure. B: AChI (Acetylcholinesterase) – propoxur exposure in the microplate assay. Numbers in phylogenetic tree represents the bootstrap support.

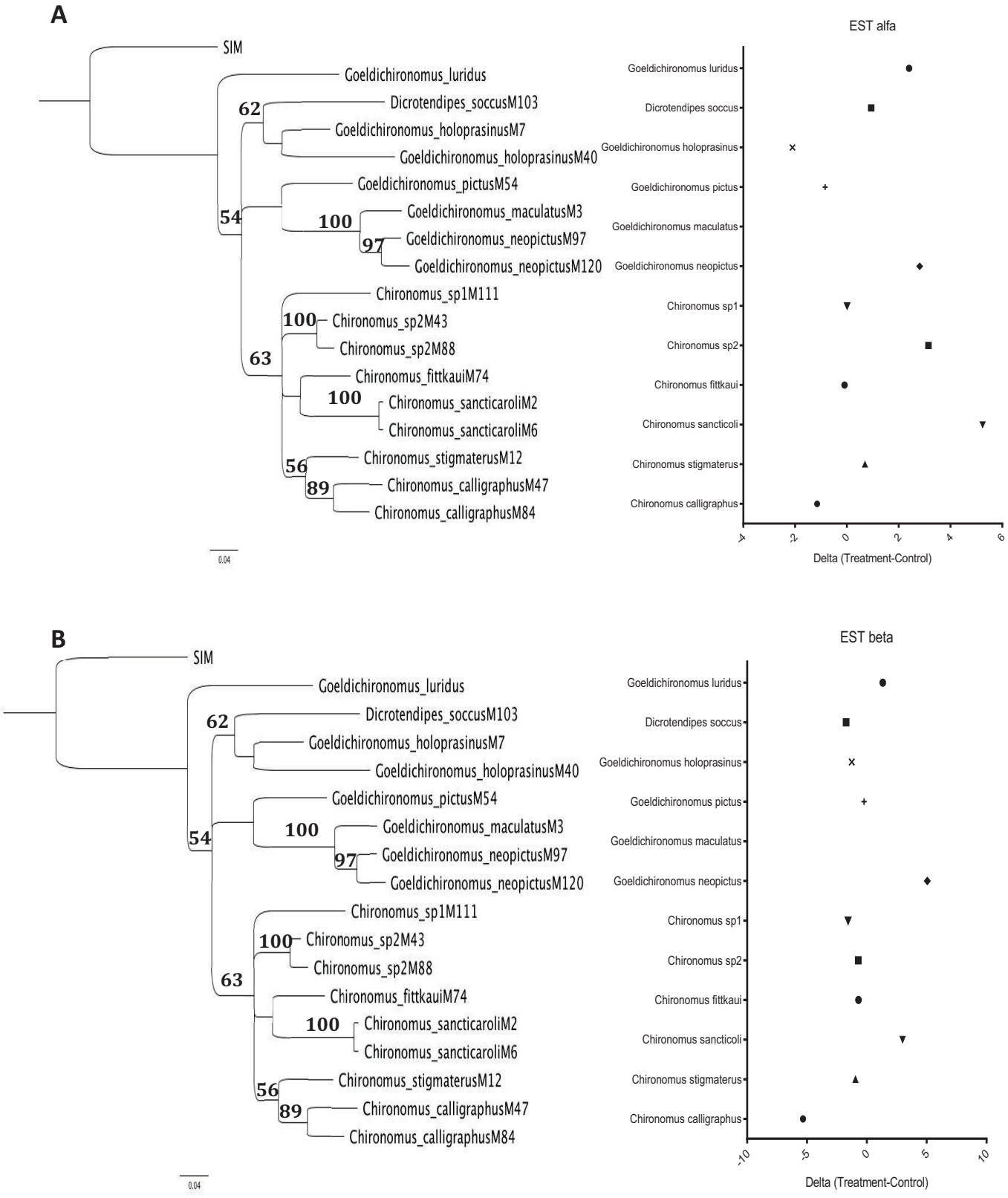

Figure 4: Phylogenetic relationships between species and enzymatic response level (treatment average-control average) of chironomids. A: EST-α (alpha-esterase) and B: EST-β (beta esterase) in malathion exposure. Numbers in phylogenetic tree represents the bootstrap support.

Looking at the activity of enzymes within the same species collected at different sites we could observe some patterns. In *G. neopictus*, for AChE from Lucélia-SP organisms, no difference between control and treatment was observed ($F_{6,8}$= 3.76; p=0.93) and the larvae from Chapecó a high level of inhibition was observed ($F_{9,9}$= 2.52; p<0.0001). The AChI assay shows different % of inhibition of the larvae from the both sites ($U_{10,9}$=12.5; p=0.0016). In the esterases similar patterns with AChE were observed increase of activity in Lucélia larvae (EST-α $U_{9,7}$= 13; p=0.0015 and EST-β $U_{9,7}$= 14; p=0.0205) and decrease of Chapecó samples (EST-α $F_{9,8}$= 4.27; p<0.0001 and EST-β $F_{9,8}$= 3.90; p=0.0007).The basal metabolism for the AChE, EST-α and EST-β, in control groups was always lower at Chapecó (AChE $U_{8,9}$= 14; p=0.0101; EST-α $F_{9,9}$= 1.82; p<0.0001 and EST-β $F_{9,9}$= 3.31; p=0.0031) (Figure 5a-d)

In *G. holoprasinus*, for AChE from Palmeira-PR organisms, no difference between control and treatment was observed ($F_{9,9}$= 2.65; p=0.14) and the larvae from Morretes-PR an inhibition was observed ($F_{14,9}$= 2.44; p=0.004). The AChI assay shows different % of inhibition of the larvae from the both sites ($F_{9,7}$=4.67; p<0.0001). In the esterases were not observed decrease of activity in Palmeira (EST-α $F_{9,9}$= 3.95; p=0.1 and EST-β $U_{9,9}$= 23; p=0.19) and decrease of Morretes samples (EST-α $F_{9,9}$= 2.20; p<0.0001 and EST-β $F_{9,13}$= 1,53; p=0.0011).The basal metabolism for the EST-α and EST-β, in control groups was different between places (AChE $F_{14,9}$= 3.37; p=0.10; EST-α $F_{9,9}$= 2.4; p=0.017 and EST-β $F_{9,9}$= 2.97; p=0.0011) (Figure 5e-h)

In *C. sancticaroli*, for AChE from both Palmeira-PR ($F_{9,9}$= 1.48; p=0.02) and LEMV colony ($F_{8,7}$= 1.32; p=0.0049) organisms, inhibition between control and treatment was observed. The AChI assay shows different % of inhibition of the larvae from the both sites ($F_{8,8}$=2.65; p=0.0004). In the EST-α, both populations had decrease to LEMV colony ($U_{9,8}$=11; p=0.0003) and increase to Palmeira ($F_{9,8}$=2.17; p=0.0041). In the EST-β were not observed decrease of activity in LEMV colony, just a trend ($U_{8,9}$= 28; p=0.18) in Palmeira we observed an increase ($U_{8,9}$= 11; p=0.0078).The basal metabolism for EST-β in control groups was different between places being LEMV colony higher (AChE $F_{9,8}$= 1.32; p=0.94; EST-α $F_{8,8}$= 2.77; p=0.56 and EST-β $U_{8,8}$= 11; p=0.0078) (Figure 5i-l).In *C. calligraphus*, for AChE from both Morretes ($F_{8,8}$= 1.36; p<0.0001) and Lucélia ($F_{9,9}$= 1.36; p=0.0027) organisms, inhibition between control and treatment was observed, not for Antonina (p=0.014). The AChI assay shows different % of inhibition of the larvae from the sites

($F_{2,13}$=2.83; p=0.0046). In the esterases, we had inhibition only to Morretes organisms (EST-α $F_{8,3}$= 2.12; p<0.0001 and EST-β $F_{8,3}$= 1,16; p=0.0441) and not to Antonina (EST-α p=0.92 and EST-β p=0.06) e Lucélia (EST-α $F_{8,9}$= 1.063; p=0.27 and EST-β $F_{9,8}$= 3.41; p=0.47). The basal metabolism for AChE and EST-β in control groups was different between populations (AChE p=0.003; EST-α $F_{2,12}$= 0.09; p=0.27 and EST-β $F_{2,12}$= 0.01; p=0.0095) (Figure 5m-p).In *C. stigmaterus*, for AChE from Chapecó organisms, no difference between control and treatment was observed ($F_{8,7}$= 2.43; p=0.48) and the larvae from Morretes-PR an inhibition was observed ($U_{7,6}$= 0; p=0.0003). The AChI assay shows different % of inhibition of the larvae from the both sites ($F_{7,4}$=2.20; p=0.0046). In the EST-α were observed decrease of activity in Morretes ($F_{8,3}$=1.03; p=0.0027) and increase of Chapecó samples ($F_{8,7}$= 1.98; p=0.0052).For EST-β, inhibition was observed only in organisms from Morretes ($U_{7,8}$=13; p=0.02) e not for Chapecó ($U_{8,8}$=27; p=0.258). The basal metabolism for AChE, in control groups was different between places (AChE $F_{8\text{-}7}$= 1,56; p=0.02; EST-α $F_{8,7}$= 1.61; p=0.08 and EST-β $F_{8,7}$= 3,29; p=0.079) (Figure 5q-t).

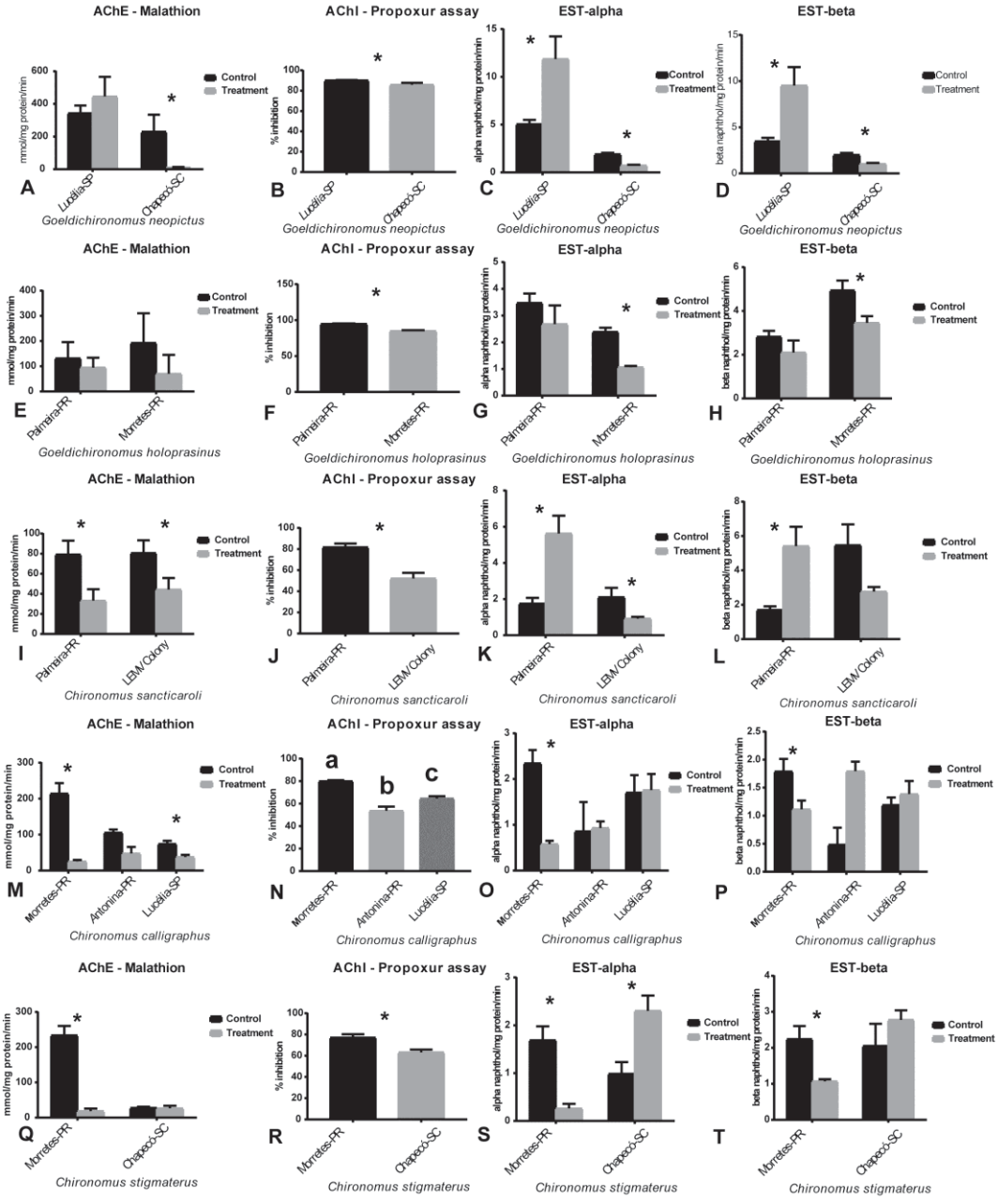

Figure 5: Enzymatic responses (AChE, AChI, EST-α and EST-β) of same species of Chironomidae collected in different sites. A-D: *Goeldichironomus neopictus,* E-H: *Goeldichironomus holoprasinus,* I-L: *Chironomus sancticaroli,* M-P: *Chironomus calligraphus* e Q-T: *Chironomus stigmaterus.* Values are expressed as mean ± standard error of mean. Asterisks/or letters indicate statistically significant differences ($p < 0.05$) compared to control group or across species; Student t-test/or Mann-Whithney/or Kruskal-Wallis.

4 Discussion

Physiological traits are often neglected in traits-based analysis in environment sciences (Poteat et al., 2015). We sought out an approach still not explored in

aquatic toxicology to comparatively investigate the biochemical responses using a phylogenetic context.

The reduction/degradation of malathion concentrations probably has caused in no alterations in AChE for *G. luridus, G. pictus* and *Chironomus* sp2, even with the high initial concentration (0.056 $mg.L^{-1}$). For *Chironomus sancticaroli* two studies showed an inhibition of AChE exposed to malathion, one of them to higher concentrations (Rebechi et al., 2014) and another one to the same concentration of the present study (Rebechi, 2016). Other studies showed AChE inhibition in chironomids exposed to organophosphates as well (Rakotondravelo et al., 2006; Printes et al., 2007; Jin-Clark et al., 2008).

Esterases had different behaviors across species, or increase of its activity or decrease. Interestingly, where EST-α and EST-β were altered both had same behavior, decrease (*G. holoprasinus* and *C. stigmaterus*) or increase (*G. neopicutus*). Some species had responses just for one esterase (increase or decrease). Esterases already showed a decrease of their activity in organophosphates exposure in chironomids (Turchetto et al., 1981; Rakotondravelo et al., 2006; Rebechi et al., 2014) but also was reported induction of their activity by other pesticides (Terriere, 1984, Montella et al., 2012). The species *G. luridus* and *G. pictus* had not significant effect to any enzyme.

Rebechi (2016) found a reduction of 25% of malathion concentration three hours after the exposure beginning, may have occurred this in our experiments leading into malathion levels not toxic to some species. In contrast, these species can be resistants to this insecticide.

Regarding propuxur assay in the microplate, almost all species had similar levels of inhibition, but the statistical analysis showed a trend separating *Goeldichironus* and *Chironomus* genera. This fact shows us that *Goeldichironomus* is more sensitive when compared with *Chironomus.* But both genera are considered the most abundant in environments with anthropic degradation (Fulan and Anjos, 2015). Furthermore, for AChE in malathion bioassays were observed more remarkable differences across species, but not here in propoxur assay. Maybe the differences in the sensitivity of species would be related to variations in the uptake rates as observed in other aquatic insects exposed to metals (Buchwalter et al., 2008; Poteat et al., 2013).

Taking in count evolutionary analysis, there was a phylogenetic signal in

AChE and EST-α for *Chironomus* but was not considered significant. Traits that are important to organisms to thrive in the environment are generally conserved (Webb et al., 2002). Enzyme activity is a trait that even within the same species, the values can be highly variable, but according to Poteat et al. (2015) phylogenetic signal can be detected in these type of traits. Analyzing the digestive enzymes across bird species Ríos et al. (2014) suggest that the differences between species in enzymes activities may be reflected by the combination of phenotypic plasticity and genetic constraints.

We tested phylogenetic to *Chironomus* and to family level, but the variation among taxonomic groups can limit the general extrapolations across species, particularly at the family level (Lenat and Resh, 2001). According to Poteat et al. (2015) the lack of clarity in these findings probably is a result of three reasons, handling with highly variable data; speciose groups, like Chironomidae (Carew et al., 2011) or whether phylogenetic signal in these traits generally breaks down at much finer levels of taxonomic resolution. Münkemüller et al. (2012) state that phylogenetic metrics have more strength when applied in a set with more than 20 species. Probably we could not detect the phylogenetic signal in *Chironomus* due to the number of species (6).

Comparing the responses of same species collected at different sites we could observe a strong influence of selection pressure to enzymatic responses. It was possible to taking a look at five different species that showed in general similar patterns. Analyzing AChE, in *G. neopictus* from two places with strong agricultural influence Lucélia-SP (sugarcane) and Chapecó-SC (soybean and wheat) organisms from the first one were tolerant (no inhibition) while SC organisms had high inhibition. Similarly comparing *G. holoprasinus* between Palmeira-PR (small farms) and Morretes-PR (costal zone, close to streams) and *C. stigamaterus* from Chapecó-SC and Morretes-PR. *C. calligraphus* from Morretes-PR and Lucélia-SP both had inhibition. *C. sancticaroli* from LEMV colony (9 years in the lab) and Palmeira-PR both had inhibition in same levels, showing that even being 9 years without exposure, the magnitude of responses is quite similar between lab and wild populations.

AChE insensivity is a frequent resistance mechanism in insects caused by a mutation at *ace* gene (Weill et al., 2002). The mutations introduce amino acids substitutions bigger than the wild-types correspondent residues and are located within the enzymes' active site, close to the catalytic triad, these substitutions block

the access by different types of inhibitor (Walsh et al., 2001). Beyond conformational process, quantitative modifications can result in AChE insensivity, like increase of amounts of this enzyme (Fournier and Mutero, 1994).

Comparing basal metabolism between controls in *G. neopictus, C, calligraphus* and *C. stigmaterus,* is remarkable to note organisms from sites with intense selection pressure (Lucélia-SP or Chapecó-SC) had lower levels of AChE activity. Natural amplification or mutation of the Drosophila AChE gene results in insecticide resistance, which is an inherited response to environmental exposure (Fournier et al., 1989). It was not clear the reduced basal levels in resistant populations, but probably the selection pressure made transgenerational effects in AChE amount in discordance with Fournier and Mutero (1994) showed in their results. Looking at AChI assays, we could observe which populations are more tolerant, as expected organisms from sites with more selection pressure had lower level of inhibition. The most intriguing was the comparison between *C. sancticaroli* from the LEMV colony was more tolerant than wild population, propably because the colony organisms were collected 9 years ago from the Curitiba metropolitan region (Capital of Paraná State). The selection pressure is more intense in this region and the organisms still not recovered the susceptible status.

Looking at esterases, in general EST-α and EST-β had similar behaviors as decrease of activity in sites with less anthropic influence and increase of their activity in sites of intense selection pressure. Esterases (carboxylesterases) behavior is variable throughout of biological groups, but their inhibition occur because the catalysis of substrates involves binding, formation of tetrahedral intermediate and hydrolysis to recovery free enzymes. In some orgonophosphates substrates, the dephosphorylation is very slow making the enzyme inactive. Malathion is a proinsecticide that can be activated by oxidative desulfuration or inactivated by hydrolysis at one of two carboxylester of their structure.

Susceptible insects are predisposed to activation of malathion rather than detoxification, theoretically due to lower titers of carboxylesterases (Motoyama and Dauterman, 1974; Wheelock et al., 2005). Insecticide resistance also may be attributed to esterases, being the probable causes the increase of gene amplification, increase of catalytic efficiency or mutant carboxylesterases. About metabolic resistance, the enhanced transcription result in an over expression of these enzymes and the capacity of insecticide metabolism is increased in resistant insects

(Wheelock et al., 2005). We observed an increase of esterases in organisms from sites with intense pressure, according to Cui et al. (2015) that increase is the main responsible for resistance status when compared with qualitative changes in the enzymes.

As observed to AChE activity between controls had been different comparing agricultural strong influence and less pressured sites. Even though, agricultural sites had an increase of ESTs activity their basal metabolism in controls was always lower. The same effect observed in AChE is present here, probably being a heritage of pressured organisms. In ESTs from LEMV colony we had the basal levels higher than Palmeira-PR, and inhibition of activity when exposed to malathion. Considering these enzymes, the laboratory organisms are still susceptible.

It is important to remark that chironomids are non-target organisms to insecticides and we found organisms with resistant status. According to Raymond et al. (2001) the fitness of animals can decrease with resistance, chironomids have an important ecological role in aquatic ecosystems, probably the populations are suffering intense selection pressure and monitoring and management steps should to be done to avoid effects to long-term.

We chose these three enzymes because AChE alone was not considered an appropriate biomarkers to evaluate resistance due to increased affinity of some organophosphates to ESTs over AChE (Rickwood and Galloway, 2004). It was possible to observe that same species collected in different sites and using organisms that had not contact with insecticides (from eggs) showed different responses. This fact demonstrates the selection pressure is an important driver of AChE and ESTs in chironomids but the acquired status can be inherited leading an important lineage context. Furthermore, in ecotoxicological studies comparisons between same species from different sites/countries even in acclimated organisms must to be done with caution, because the responses are also the reflection of the lineage history.

5 Conclusions

It is possible to observe that selection pressure is a main driver of the responses of the AChE and ESTs in chironomids, but more studies can investigate if there is some evolutionary contribution at least to *Chironomus* genus. *Goeldichironomus* in general is more susceptible than *Chironomus* according with AChI assays but some uptake/or efflux/other biochemical pathways mechanisms can influence the tolerance of organisms. Enzymatic activity between control groups shows us transgenerational pressure can decrease the titers of AChE and ESTs, the biological meaning is still unclear. The natural populations of chironomids have been suffering the pressure of insecticides demonstrated by resistant status of some animals.

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CONSIDERAÇÕES FINAIS

Mesmo larvas de Chironomidae sendo consideradas organismos tolerantes a degradação ambiental, o presente estudo demonstra sua sensibilidade em nível subletal mesmo em concentrações ambientalmente relevantes de xenobióticos.

Quando *Chironomus sancticaroli* foi exposta a sedimentos provindos de diferentes áreas de influência antrópica, as larvas apresentaram alterações em todos os tratamentos. Um exemplo foram as amostras do Rio Santo Anastácio (Agricultura) que apresentaram alteração para quase todos os biomarcadores testados, além disso a enzima Superóxido-dismutase (SOD) teve uma inibição para quase todos tratamentos, causados provavelmente pelos altos níveis de espécies reativas de oxigênio. Além disso, estresse oxidativo pôde ser evidenciado em amostras do Rio do peixe (Em industrialização) e Rio Santo Anastácio (Agricultura) devido a presença de maiores concentrações de lipoperóxidos. Em relação a sensibilidade tecidual, o corpo gorduroso visceral foi o órgão mais sensível, demonstrando vacuolização do citoplasma e redução no volume celular. Mesmo ambientes considerados de destino à conservação, mostraram toxicidade subletal para os organismos, o que torna de grande dificuldade a identificação de áreas referência para estudos ecotoxicológicos.

Quando *Chironomus dilutus* foi confrontada com a contaminação por metais e baixos níveis de oxigênio dissolvido, os organismos não apresentaram mortalidade, porém tiveram respostas em nível molecular e fisiológico. As hemoglobinas se mostraram multifuncionais, mas nem todas as que foram testadas responderam a hipóxia, como esperado para essas globinas. Algumas delas responderam somente quando em combinação arsênio + hipóxia (HbC e D). A HbA respondeu somente ao arsênio, tendo talvez uma função no metabolismo. Além disso a enzima GST só apresentou resposta quando nas combinações, interessantemente influenciada pela hipóxia. O consumo de oxigênio também foi aumentado nas larvas expostas ao arsênio.

De um ponto de vista comparativo, quando 12 espécies de Chironomidae distribuídas em 3 gêneros, foram expostas a uma mesma concentração de malathion e propoxur e a variável era a unidade espécie foram observados que cada qual tinha um nível diferenciado de resposta das enzimas mensuradas. Essa variação ao longo das espécies foi comparado em uma abordagem filogenética, e

não foi detectado sinal filogenético nas enzimas AChE, EST-α e EST-β para todas as espécies, já para o gênero *Chironomus* existiu uma tendência de sinal filogenético nas enzimas AChE, EST-α que a análise não foi sensitiva para detectar. Possivelmente a pressão de seleção é o principal direcionador da tolerância nesses organismos, pois espécies coletadas em locais diferentes que sofrem forte pressão de seleção apresentaram respostas bioquímicas diferenciadas, podendo alguma ser consideradas resistentes.

Por fim é possível afirmar que os organismos da família Chironomidae possuem uma considerável elasticidade fisiológica que permite-os sobreviver nesse ambientes desafiadores, porém alterações foram detectadas que podem refletir em algum custo no fitness das espécies a longo prazo podendo causar alterações na polulações.

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